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DNA-binding and DNA-protection properties of novel 1,4- dihydropyridine derivatives and nucleoside analogues

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SUMMARY

DNA interaction with mutagenic compounds leads to undesirable transformation of DNA molecule and DNA damage. Ineffective repair of these lesions become a reason of inception and pathogenesis of various diseases, for example *diabetes mellitus*, neuropathies and cancer. The prevention of DNA damage and stimulation the capacity of DNA repair could be a good treatment strategy or prophylaxis of various diseases. Recently several data indicated about the ability of some antioxidant and antimutagenic compounds directly interact with DNA without any mutagenic and cancerogenic insult. The aim of our study was to determine the DNA binding properties for a series of 1,4-dihydropiridines (1,4-DHPs), to examine the interaction modes for the most active binders of 1,4-DHPs, to define their capability to scavenge peroxynitrite, to determine the ability to bind proteins, to assess the capability of 1,4-DHPs to protect DNA against oxidative stress and pro-apoptotic DUX 4 protein expression produced damage. The DNA, protein and peroxynitrite binding properties of 1,4-DHPs were investigated using fluorimetry assay, circular dichroism and UV/VIS spectroscopy. The ability to protect DNA in living cells against oxidative stress and DUX4 protein negative impact the single cell gel electrophoresis (Comet Assay) was used for these purposes. Obtained data revealed that some of 1,4-DHPs have a high affinity to DNA, especially salts of AV-153, PP-544- NH_4, cerebrocrast and tauropyrone. The capacity of interaction depends on groups in different positions of 1,4-DHP ring. Metal ions in position 4 modify DNA-binding and DNA-protecting effects of the AV-153 salts. Salts of AV-153 are capable to bind human serum albumin. Some of 1,4-DHPs with DNA binding properties are good protectors against peroxynitrite induced oxidative damage, especially Na and Ca salts of AV-153 and cerebrocrast. AV-153-Na significantly enhanced the repair kinetic after peroxynitrite induced damage. Based on the obtained results, it may be suggested that direct binding of antimutagens to DNA could be a possible DNA-protecting mechanism of action.

Keywords: 1,4 – dihydropiridines, AV-153 salts, DNA binding, antimutagens, antioxidants, peroxynitrite, DNA repair, DNA protection

KOPSAVILKUMS

Dažu savienojumu mijiedarbība ar DNS ir saistīta ar nevēlamām DNS molekulas struktūras izmaiņām, kas noved pie DNS bojājumiem. Neefektīva DNS bojājumu reparācija var kļūt par iemeslu dažādu slimību patoģenēzei, piemēram, cukura diabēts, neiropātijas vai kanceroģenēze. DNS pasargāšana no bojājumiem, kā arī DNS reparācijas kapacitātes stimulēšana varētu kļūt par efektīvu ārstēšanas stratēģiju vai dažādu slimību profilaksi. Nesen iegūtie dati liecina, ka daži savienojumi, kuriem piemīt antioksidatīvas un antimutagēnas īpašības, spēj tieši mijiedarboties ar DNS molekulu. Šī pētījuma mērķis bija izpētīt dažādu 1,4-dihidropiridīna (1,4-DHP) atvasinājumu spēju mijiedarboties ar DNS, noteikt mijiedarbības veidu, pārbaudīt spēju mijiedarboties ar proteīniem, novērtēt 1,4-DHP atvasinājumu spēju reaģēt ar peroksinitrīta molekulu un papildus novērtēt 1,4-DHP spēju pasargāt DNS integritāti oksidatīvā stresa apstākļos, kā arī no pro-apoptotiskā DUX4 proteīna ekspresijas izraisītajiem bojājumiem. 1,4-DHP spēja mijiedarboties ar DNS, proteīniem un peroksinitrītu tika izpētīta, izmantojot fluorimetriskās metodes, cirkulāro dihroismu, infrasarkanās un ultravioletās/redzamās gaismas spektrofotometriju. Antioksidatīvās īpašības neizpētītās sistēmās tika izvērtētas, izmantojot vienšūnas gēla elektroforēzi sārmainos apstākļos, jeb Komētu testu. Iegūtie dati pierādīja, ka daži 1,4-DHP spēj intensīvi un dažādi mijiedarboties ar DNS, it īpaši AV-153 sāļi, PP-544-NH4, cerebrokrasts un tauropirons. Metāla joni 4.pozīcijā modificē AV-153 sāļu mijiedarbības intensitāti ar DNS, mijiedarbības veidus un antioksidatīvās īpašības. Tika pierādīts, ka AV-153 sāļiem piemīt īpašība mijiedarboties ar cilvēka seruma albumīnu. Daži 1,4-DHP atvasinājumi, kuriem piemīt mijiedarbības spēja ar DNS, labi pasargāja šūnu DNS no peroksinitrīta un DUX4 proteīna izraisītajiem bojājumiem. AV-153 nātrija un kalcija sāļi, kā arī cerebrokrasts parādīja sevi kā visefektīvākie savienojumi šajā jomā. AV-153-Na ievērojami paātrināja reparācijas ātrumu pēc peroksinitrīta izraisītajiem bojājumiem un paaugstināja reparācijas enzīmu aktivitāti. Pamatojoties uz iegūtajiem rezultātiem, var pieņemt, ka antimutagēnu mijiedarbība ar DNS varētu būt iespējams DNS pasargāšanas mehānisms.

Atslēgvārdi: 1,4-dihidropiridīni, AV-153 sāļi, mijiedarbība ar DNS, antimutagēni, antioksidanti, peroksinitrīts, DNS reparācija, DNS pasargāšana

CONTENTS

ABBREVIATIONS

1,4-DHP – 1,4 dihydropirydines
3HF – hydroflavone
5-FoUra - 5-formauracil
5-OHMeUra - 5-hydroxymethyuracil
8-oxodA - 8-oxodeoxyadenosine
8-oxodG- 8-oxo-7,8-dihydro-2`-deoxyguanosine
8-oxoG – 8-oxoguanine
Å - angstrom
A-adenine
ActD – actinomycin D
ADP – adenosine diphosphate
AFB1- aflatoxin B1
AMP – adenosine monophosphate
AO – acridine orange
AOA – antioxidant activity
AP – apurinic
APE1 – human AP – endonuclease 1
ARA – antiradical activity
AV-153 - Sodium 3,5-bis-ethoxycarbonyl-2,6,dimethyl-1,4-dihydropyridine-4-carboxylate
AU – arbitrary units
BER – base excision repair
Bp – base pair
CCB –calcium channel blocker
C-cytosine
CD – circular dichroism
CFTR – cystic fibrosis transmembrane conductance regulator
CNS(1) chrom-(neocarzinostatin chromophore)
CNS(2) central nervous system
CPQE - chloroquinoline derivatives 1-(2-chloro-4-phenylquinolin-3-yl)ethanone
ct- DNA – calt thymus DNA
DCPQE - 1-(2,6dichloro-4-phenylquinolin-3-yl)ethanone
DGs - glycosilases
DM – diabetes mellitus
DMEM - Dulbecco`s Modified Eagle Medium
DMSO – Dimethyl Sulfoxide
DNA - Deoxyribonucleic acid
DPPH a.o. – stable free radicals
dRP – 5`deoxyribose phosphate termini
DSB – double strand break
DUX4 - Double homeobox 4
EDTA - Ethylenediaminetetraacetic acid
eNOS – endothelial nitric oxide synthase
EtBr – Ethidium bromide
FapyA - 4,6-diamino-5-formamidopyrimidine
FapyG - 2,6-diamino-4-hydroxy- 5-formamidopyrimidine
RNS – reactive nitrogen species
ROS – reactive oxygen species
RS – reactive species
FEN1 – flap endonuclease 1
GABA – gamma-amilobytiric acid
G-guanine
GMP – guanosine-5´-monophosphate
$H^{\cdot}$ - hydrogen atom
H_2O_2 - hydrogen peroxide
HCC-hepatocellular carcinoma
HEH – Hantzsch ester
HEPES - 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid
HGPRT – hypoxanthine guanine phosphoribosyl transferase
HSA – human serum albumine
iNOS – inducible nitric oxide synthase
Iz – imidazole – 4- one
K_b – binding constant
K_{sv} – Stern Volmer constant
kDNA-kinetoplast deoxyribonucleinic acid
LDL- low density lipids
$LOO^{\cdot}$ - lipid peroxides
LOSI – Latvian Institute of Organic Synthesis
LP-BER – long path base excision repair
LTCC – L-type calcium channel
MCMQC - methyl 2-chloro-4-methylquinoline-3-carboxylate
MDCPQC - methyl 2,6-dichloro-4-phenylquinoline-3-carboxylate
MPP^+ - 1-methyl-4-phenylpyridinium
mtDNA – mitochondrial DNA
n – binding site
Na_2EDTA - disodium ethylenediaminetetraacetate dihydrate
NaC – nucleus accumbens
NADH – nicotineamide adenine dinucleotode
NADPH –nicotinamide adenine dinucleotide phosphate
NEIL(1,2,3,4)
NER – nucleotide excision repair
NMR – nuclear magnetic resonance
NO – nitric oxide
NTH1 - endonuclease III-like 1
O_2^- - superoxide anion radical
OGG1 - 8-oxoguanine DNA glycosylase
$OH^{\cdot}$ - hydroxyl radical
$ONOO^-$ - peroxynitrite
$ONOOCO_2^-$ - nitrosoperoxycarbonate
ONOOH - peroxynitrous acid
Oz – 2,24 – triamino – 5(2H)- oxalozone
PARP1 – poly [ADP-ribose] polymerase
PBS – phosphate buffered saline
PI-propidium iodide
RF-C – replication factor - C
RNA – ribonucleic acid

SAR – structure activity relationship
SSB – single strand break
STZ – streptozotocine
TRIS - tris(hydroxymethyl)aminomethane
T-thymine

INTRODUCTION

Synthetic derivatives of 1,4-dihydropiridine (1,4-DHPs) possess valuable biochemical and pharmacological activities including genome –protecting effects. Some representatives of 1,4-DHPs group demonstrate antimutagenic, anticlastogenic activity and stimulate DNA repair (Goncharova et al. 2001, Vartanian et al. 2004, Ryabokon et al. 2005). Other compounds of the group produce modulating effects on cardiovascular and neuronal processes and manifest anticancer, geroprotective and many more effects (Khedkar and Auti 2013). 1,4-DHPs also act as a free radical scavengers and react with peroxynitrite (Lopez-Alarcon et al. 2004, Lob et al. 2006). Most biological effects of this class of the compounds are usually described as calcium channels blockers. However, our attention was focused on 1,4-DHPs synthetized in the Latvian Institute of Organic Synthesis and considered to be `unusual`. These compounds do not block calcium channels or are very weak Ca-channel binders. It was recently revealed that a representative of this group AV-153-Na possesses antioxidant and DNA repair enhancement activities (Ryabokon et al. 2005). Moreover, it was reported that AV-153-Na is able to interact with DNA, possibly in a place of single strand break between two pyrimidines (Buraka et al. 2014). This finding opens the question about the mechanism of action of some 1,4-DHPs derivatives with a perspective to reveal general mechanism of antimutagenic effects. Our results revealed structure-functional relations of DNA-binding capacity and mode of binding of the most active compounds. Interestingly, DNA binding properties of some derivatives of 1,4-DHP were coherent with their DNA protective activity. Moreover, we have observed significant differences in DNA binding, peroxynitrite scavenging capacities and DNA protective effects against oxidative damage of different AV-153 salts.

AIM OF THE STUDY

The aim of study the was to determine the DNA binding activity and the binding mode of water soluble and lipophilic derivatives of 1,4-DHPs and to evaluate the significance of modifications of the molecule on DNA binding capacity and binding mode and to evaluate antioxidant activity of 1,4-DHP by testing peroxynitrite scavenging properties and the capability to protect DNA in living cells against oxidative stress.

TASKS TO REACH THE AIM

1. To test the affinity of different water soluble and lipophilic derivatives of 1,4-DHP to DNA using different spectroscopic approaches
2. To assess the possible DNA interactions modes of the most active 1,4-DHPs applying fluorescence spectroscopy
3. To define the preferable interactions of AV-153-Na with DNA bases using UV/VIS spectroscopy
4. To determine the ability of 1,4-DHPs to bind human serum albumin using different spectroscopic approaches
5. To test the capability of water soluble and lipophilic 1,4-DHPs to scavenge peroxynitrite applying kinetic study using UV/VIS spectroscopy
6. To study the ability of 1,4-DHPs to protect DNA in living cells against peroxynitrite and pro-apoptotic DUX4 protein damage and enhance DNA repair after peroxynitrite treatment using single cell gel electrophoresis (Comet Assay)

THE HYPOTHESIS

1,4-DHP penetrate the cell, bind DNA and trigger DNA repair.

MAIN THESIS OF DEFENCE

1. Some representatives of 1,4-DHPs have a high affinity to DNA which depends on modification of groups in position 3,4 and 5
2. 1,4-DHPs interact with DNA via intercalation and groove binding, the binding mode depends on structural features of compound
3. Some representatives of 1,4-DHPs with a high affinity to DNA are efficient DNA protectors against oxidative stress

Experimental part of work has been developed in Latvian Institute of Organic Synthesis and Faculty of Medicine, University of Latvia

1. LITERATURE REVIEW

1.1 Ligand interaction with DNA

The most important biological systems and processes are dependent on reversible interactions of one molecule with another. DNA is the main target for the variety of molecules. The nature of interaction between DNA and small ligands is very important because it may significantly influence DNA transcription, replication and gene expression, and thereby influence physiological functions of the cells (Zhu et al. 2014). Ligands can bind to DNA molecule in various ways from irreversible covalent to simple electrostatic interactions (Rocha 2015).

1.2 DNA-ligand complex: formation of chemical bond

Supramolecular chemistry is a field of chemistry that comprises various phenomena of physical, biological and chemical processes. Molecular recognition through different ways of molecular interactions is one of them. Hydrogen bonding interaction, $\pi - \pi$ interactions, electrostatic interactions and van der Waals interactions are very important driving forces for ds-DNA stability and DNA-ligand recognition (Gonzalez-Ruiz et al. 2011). Interacting molecules determine different modes of binding, comprising covalent binding and non-covalent interactions. Generally, small molecules bind to DNA via three principal ways, (Fig. 1.1) i.e., (i) outside-edge binding or electrostatic attraction with anionic DNA backbone (ii) intercalating between base pairs and (iii) major and minor groove binding (Sheng, Gan, and Huang 2013, Almaqwashi et al. 2016). The interaction mode depends on DNA and molecule structural features and many compounds have an ability to interact with DNA at different modes simultaneously. Intercalation and specific interactions with major and minor grooves are the strongest binding modes. Nevertheless, electrostatic interactions have a high significance. The stability and conformation of DNA requires binding with positively charged counterions (Lipfert et al. 2014).

The study of interaction of small molecules with DNA is helpful for understanding of the mechanism of interaction and for design of new highly efficient drugs, which can alter and/or inhibit the functioning of DNA.

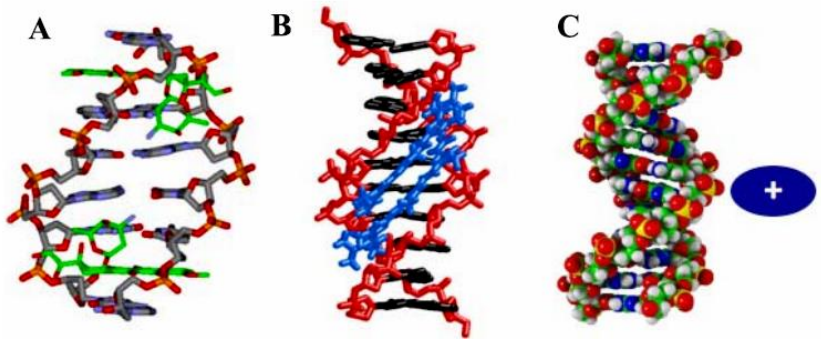


Figure 1.1. **Three principal ways of non-covalent interaction with DNA.** (A) Intercalation (B) groove binding (C) outside-edge binding/electrostatic interaction (Gonzalez-Ruiz et al. 2011).

1.2.1 Covalent binding

Covalent binding of drugs is quite critical for a proper functioning of DNA since almost all-important processes in DNA are completely inhibited. Platinum-based drugs are widely used in chemotherapy and are the most studied compounds that interact with DNA by forming covalent bonds (Rocha 2015, Sheng, Gan, and Huang 2013, Salerno et al. 2016).

The inorganic drug cisplatin (CIS; cis-diammidedichloroplatinum (II), Peyrone`s salt) was the first representative of platinum containing anticancer drugs. Nowadays cisplatin and its analogues (Fig. 1.2) oxaliplatin (oxalato-1,2-diaminocyclohexane platinum(II)) and carboplatin (cis-diammine(1,1-cyclobutane-dicarboxylato)platinum(II)), which are less toxic are the most frequantly used anticancer drugs against several solid tumors, such as ovarian, neck and head , testicular, cervial, prostate, brains and lung cancers. (Ho, Au-Yeung, and To 2003, Rocha 2015, K.R. et al. 2014). These are also effective against lymphomas, carcinomas and sarcomas (Dasari and Tchounwou 2014).

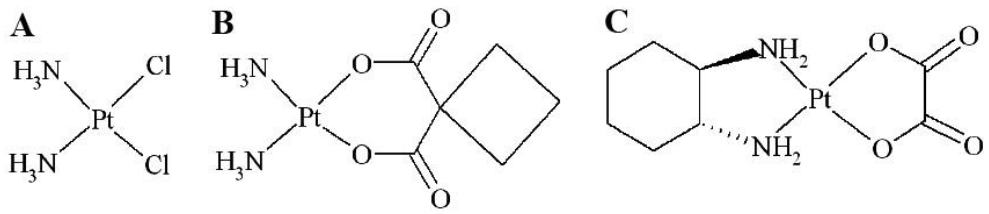


Figure 1.2. **Chemical structure of cisplatin and its analogs** (A) Cisplatin (B) Carboplatin (C) Oxaliplatin

All these platinum complexes form stable adducts with DNA. Pt-DNA adduct formation results in formation of various types of crosslinks and introduces perturbations in DNA structure, leading to inhibition of replication and transcription and to cell cycle arrest. Cisplatin, carboplatin and oxaliplatin have different solubility and this predetermines chemical activity and toxicity of the compounds. All of them undergo hydrolysis in the cell and become positively charged, this is a key point for forming a complex with DNA. These drugs bind to N7 position of purine bases, the major Pt binding site, forming Pt-DNA adducts. Cisplatin and oxaliplatin undergo equation much faster than carboplatin (McWhinney, Goldberg, and McLeod 2009, Hah et al. 2006). The adducts formed by these compounds may be different, 60-65% are 1,2-intrastrand d(GG) adducts, 25-30% are 1,2 intrastrand d(AG) adducts, 5-10% are 1,3-intrastrands d(GXG) adducts (Fig. 1.3) (Boulikas 2007). Moreover cisplatin interacts with different molecules in the cell, including RNA and phospholipids, however DNA is the main target of this drug (Wang, Lu, and Li 1996). Unlike cisplatin, which loses its two chloride ions simultaneously and forms diadduct with purine bases, carboplatin and oxoliplatine form monoadducts followed by diadduct formation (Hah et al. 2006).

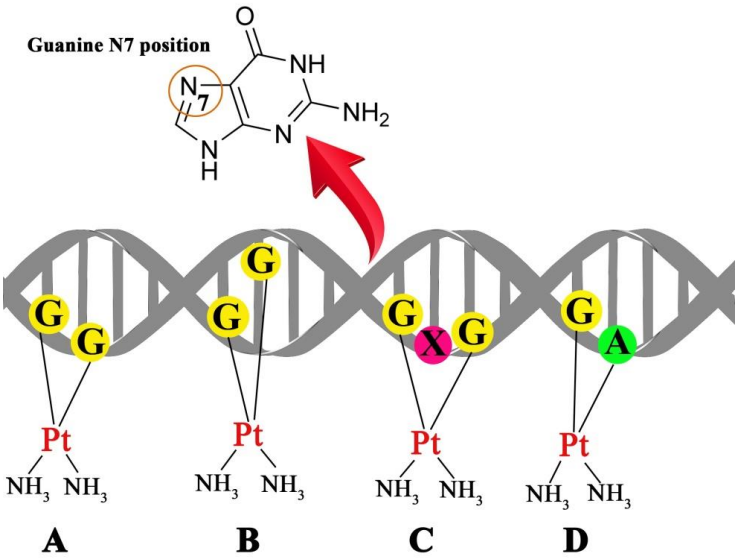


Figure 1.3. **Common types of DNA-cisplatin crosslinks** (A) 1,2-intrastrand dGG (B) 1,2-interstrand dGG (C) 1,3-intrastrands d(GXG) (D) 1,2 intrastrand d(AG)

Alkylators is another type of compounds, that contain a reactive N,N-bis-2(2-chloroethyl)amine fuctional group and covalently interact with DNA. Nitrogen

mustards, which are effective anticancer drugs, can be divided in two groups: compounds, which react directly with DNA and compounds, which form a reactive intermediate and then react with DNA. Alkylation occurs by two nucleophilic substitution reactions or SN1 and SN2 alkylation pathways (Gruppi et al. 2015). The most widely used representatives of this group are chlorambucil, cyclophosphamide and mechlorethamine (Cheung-Ong, Giaever, and Nislow 2013). These agents alkylate guanine residues in N7 position via the aziridium intermediate (Fig. 1.4)

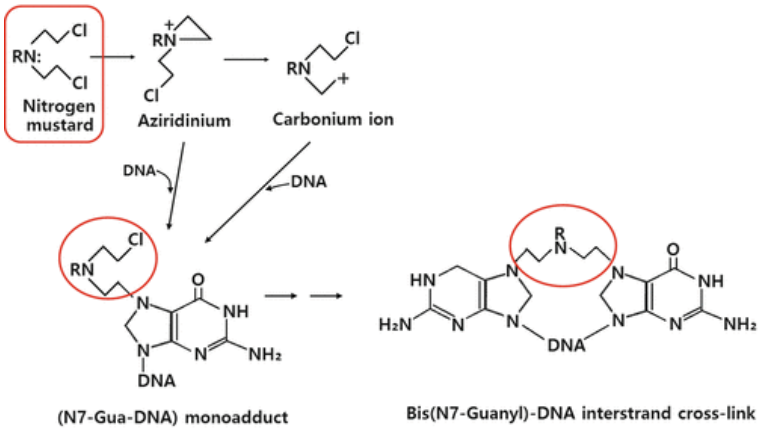


Figure 1.4. **Nitrogen mustard induced monoadduct via formation of highly reactive aziridium intermediate** (Kim et al. 2016).

Another molecule, which initiates alkylation, is aziridine antibiotic mitomycin C (Fig. 1.5A). This compound is widely used against variety of tumours. Through the minor groove mitomycin C alkylates guanine residues of 5`-CG-3` sequences unlike the nitrogen mustards, which react exclusively with guanine residues in 5`GNC-3` through the major groove (Sheng, Gan, and Huang 2013, Wang et al. 2016, Noll, Mason, and Miller 2006). To be activated, mitomycin C requires reductive activation of its quinone ring and forms reactive hydroquinone product via a variety of intermediates, elimination of methanol group leads to leuco-aziridinomitosene formation (Bass et al. 2013) and the elimination of carbamoyl group produces the hydroquinone methide intermediate that alkylates the guanine and after oxidation forms interstrand crosslinks (Noll, Mason, and Miller 2006). Azinomycin B (also known as carzinophilin A) (Fig 1.5B) also is representative of DNA-alkylating antibiotics. However, this compound does not need reductive activation.

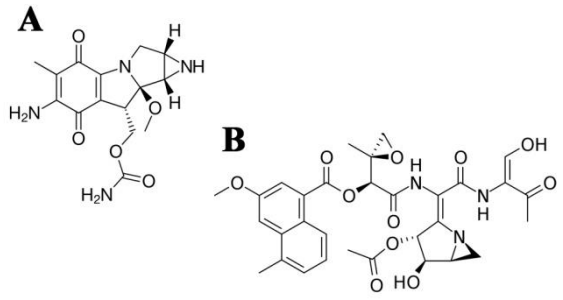


Figure 1.5. **Chemical structure of alkylating antibiotics** (A) Mitomicyn C (B) Azinomycin B

Psoralens or derivatives of 9-methoxy-7H-furo[3,2-g]chromen-7-one tricyclic ring structures are a class of furocoumarins, that are widely used in the phototherapy of skin disorders, mainly of psoriasis and vitiligo. These compounds interact with DNA via intercalation and then form covalent adducts, preferably with thymines (T) 5`-TA-3` and 5`-AT-3` sequences in the presence of ultraviolet radiation, forming cyclobutane monoadducts (Noll, Mason, and Miller 2006, Blackburn 2006, Sheng, Gan, and Huang 2013, Hermanson 2013).

Aflatoxin B1 (AFB_1) is a genotoxic compound that contaminates a variety of foods, such as corn, peanuts, rice, dried fruits and cottonseed meals. AFB1 is a hepatocarcinogen implicated in hepatocellular carcinoma (HCC) development. AFB1 is metabolically conversed by cytochrome-450 enzyme to highly reactive intermediate *exo*-8,9-epoxide, which covalently reacts with guanine bases in the liver cell DNA, inducing DNA adducts (Fig. 1.6) (Hamid et al. 2013). It was believed that the reaction of AFB_1 exo-8,9-epoxide and DNA proceed by the intercalative mode, however it was recently revealed that the binding of AFB_1 to DNA occurs throught groove binding rather than intercalation and caused by hydrogen bonding (Ma, Wang, and Zhang 2017).

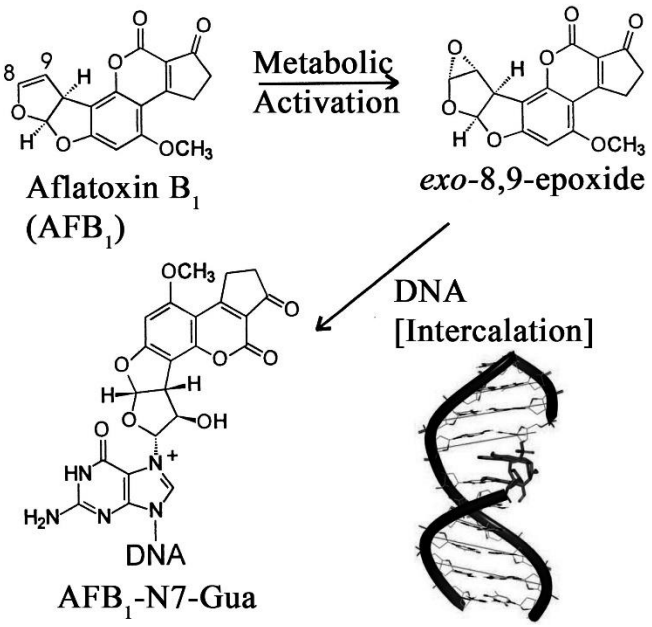


Figure 1.6. **AFB_1 binding to DNA** (Wang et al. 2016).

1.2.2 Outside-edge binding

DNA is an exceedingly charged polyanionic molecule and its polyanionic nature directly affects the interaction with compounds of opposite charge. The stability of DNA conformation and biological behaviour depends on association with multivalent metal cations from the solution, such as sodium (Na^+) or magnesium (Mg^{+2}). This process, called counter-ion condensation was developed and described by Gerald Manning in the early 1970s and defined as non-localized binding (Blackburn 2006). This binding process is referred as condensation due to cation cloud formation around the DNA and moving along the helix. The binding of cationic molecules to DNA neutralizes negatively charged phosphate groups thereby releasing condensed counter-ions. This plays an important role in binding intensity, because entropic release of counter-ion provides a free energy for groove binding and intercalation. Such binding mode is strongly dependent on salt concentration of the solution and the interaction affinity decrease under low salt concentration. For the effective DNA-drug binding, the design of molecular structure of small ligands, as a rule, includes the electrostatic component (Strekowski and Wilson 2007). Several types of molecules interact with DNA mainly through outside-edge interaction. Polyamines, spermine, spermidine, putrescine and cadaverine are the typical electrostatic interaction drugs, usually used for charge-charge effect reduction (Fig. 1.7) (Blackburn 2006). Polyamines interact

more effectively with AT-rich sequences, the binding strength depends on the charge of the compounds, increasing in the row spermine>spermidine>putrescine> cadaverine (Kabir and Suresh Kumar 2013). Spermine and spermidine the are multifunctional molecules and play important roles in several biological processes, such as DNA packaging, modulation of gene expression, activation of DNA synthesis, enzyme activation, cell differentiation and proliferation (Casero and Woster 2001, Thomas* and Thomas 2001, Wang et al. 2012). The DNA-protecting activities of spermine and spermidine are also reported, these possess an ability to protect DNA against radiation, free radicals and reactive oxygen species (ROS) (Rider et al. 2007). It is of interest that binding of these compounds induces conformational transitions of DNA and provokes conformational transition of B-DNA to Z-DNA or A-DNA (Ruiz-Chica et al. 2001).

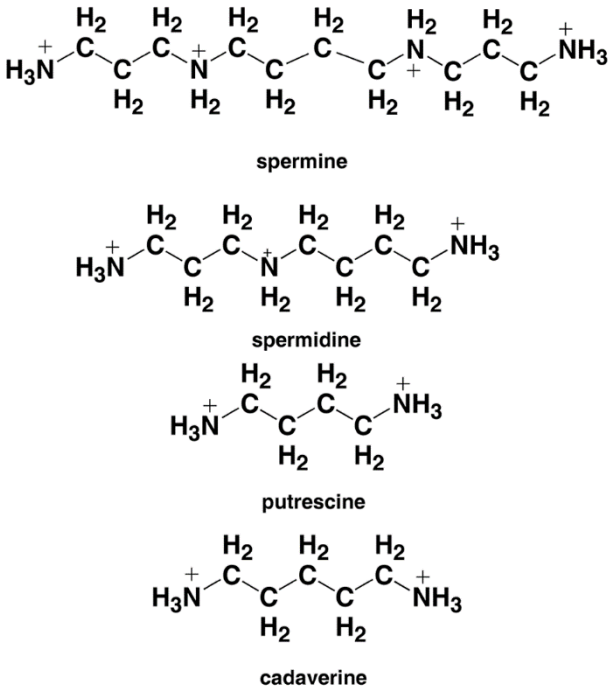


Figure 1.7. **Chemical structures of DNA binding polyamines** (Kabir and Suresh Kumar 2013).

1.2.3. Minor groove binding

Compounds that possess the ability to interact with DNA minor groove are in the spotlight of different research areas due to their high potential in treatment of cancer and various infections, as well for their application in biotechnology (D'Incalci and Galmarini 2010, Hartley 2011, Chenoweth et al. 2009, Paine et al. 2010, Wilson et al.

2008, Chavda et al. 2011, Stadler et al. 2011, Hsu and Dervan 2008). The minor groove is quite malleable when interacting with small molecules. Numerous mono-charged, multicharged and neutral ligands manifested the ability to interact with the minor groove. The structure of classic minor groove binders has a number of specific structural features; molecules consist of several aromatic rings connected by single bonds - pyrrole, benzene or furan with flat conformation, that supply flexibility, availability of positively charged groups, ability to donate H-bonds. Minor groove binders in most cases have a typical crescent shape, which is compatible with the minor groove, thus facilitating interaction as van der Waals contacts and hydrophobic interactions. (Blackburn 2006, Tanious et al. 2007, Goodwin et al. 2006, Neidle 2001) The structure of these molecules has an ability to twist and to fit into the helical structure of groove and displace water molecules. (Silverman and Holladay 2014). The most known minor groove binders are DAPI, Hoechst 33258, berenil. distamycin A, netropsin, mithramycin and pentamidine (Fig. 1.8) (Wang et al. 2016). The structure of DNA and Hoechst 33258 complex is shown in the Figure 1.8. The Hoechst 33258 is attached to the phosphate backbone in the minor groove, the compound forms strong van der Waals contacts with DNA. Minor groove binding molecules can be divided in two major groups: compounds that bind to minor groove of GC-rich sequence, for example chromomycin, olivomycin and mithramycin, which have antitumour activity (Barcelo et al. 2010) and molecules, which preferably bind AT-rich areas. Minor groove binders have anti-tumor, anti-bacterial and anti-viral activities (Barrett, Gemmell, and Suckling 2013). Berenil, propamidine and pentamidine are the representatives of aromatic diamidines with great antytrypanosomal and antiviral activity. These compounds strongly bind in the minor groove, preferably at the center of AATT DNA sequences (Nguyen et al. 2004).

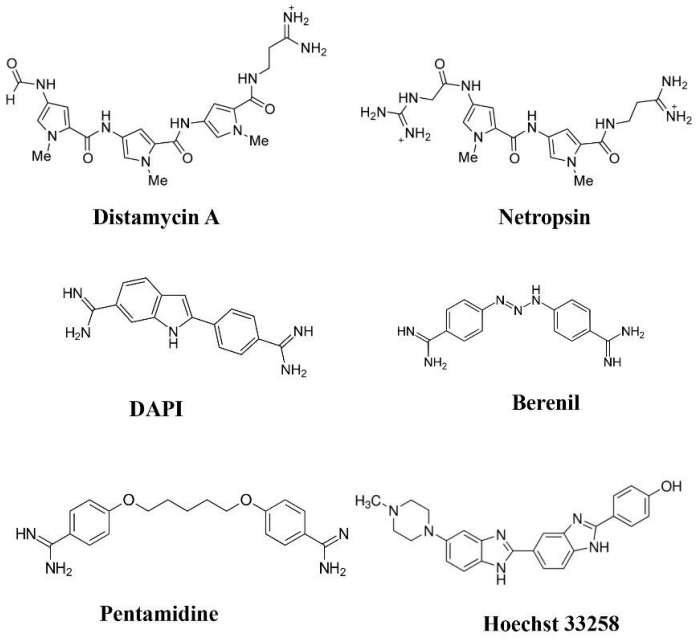


Figure 1.8. **Chemical structures of minor groove binders** (Wang et al. 2016).

AT rich minor grooves have a greater negative electrostatic potential than GC rich grooves and this leads for higher AT selectivity for cationic molecules (Paul and Bhattacharya 2012). DNA and groove binder complex are stable due to hydrophobic interactions. Moreover groove binders do not induce significant conformation changes in DNA molecule and do not change the structure of DNA free energy (Palchaudhuri and Hergenrother 2007).

Generally AT rich regions are narrower than GC rich regions, that is why AT regions are more amenable for binding of flat aromatic molecules, making optimal van der Waals contacts. (Silverman and Holladay 2014, Blackburn 2006).

Typical minor groove binders such as netropsin and distamycin A (Fig. 1.8) are natural cationic compounds, possessing three or two N-methylpyrrole rings, both are pyrrole-amidine antibiotics. These compounds prefer AT rich regions for binding. For these compounds the 5'-AATT binding site is more preferable than 5'-ATAT, but 5'-TTAA and 5'TATA sites are limited in binding. Netropsin fits symmetrically in the center of AATT region in the minor groove, displaces water molecules, forms three hydrogen bonds with N-3 of adenine and C-2 carbonyl oxygen of thymine along the groove and forms van der Waals contacts with the atoms of the sugar –phosphate

backbone of the groove. Pyrrole rings of netropsin are holded by these contacts almost parallel to the sugar-phosphate walls. The interaction of netropsin with DNA induce a slight widening of the minor groove (Fig 1.9) (Fang et al. 2010).

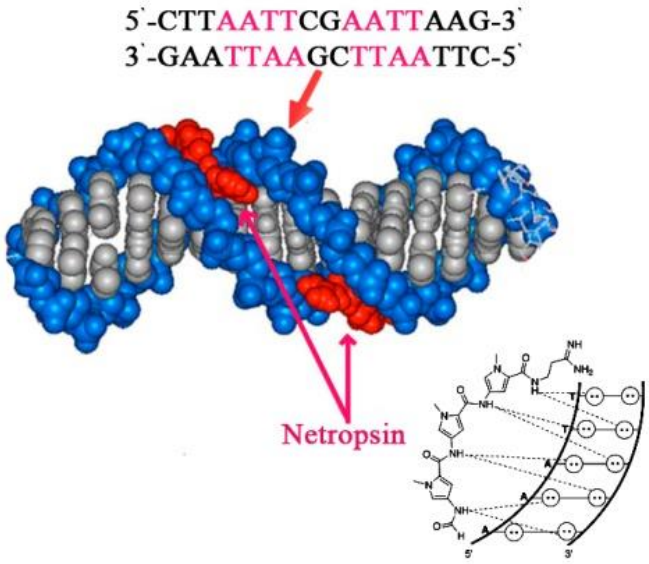


Figure 1.9 **Netropsin upon DNA minor-groove binding** (Fang et al. 2010, Blackledge and Melander 2013).

The binding affinity and mode of distamycin A with A-T regions is similar to netropsin. However, it was revealed that distamycin A has additional binding mode. Nuclear magnetic resonance (NMR) and X-ray crystallography studies showed the existence of two types of distamycin A- DNA complexes. 1:1, when one molecule fits into groove and 2:1 when two molecules in antiparallel orientation stacked side by side into groove depending on A-T base pair amount in the sequences. 1:1 complex formation occurs if sequences contain only four A-T base pairs and extends along the groove, the minor groove is compressed and the major groove is widened (Fig. 1.10A) 2:1 complex forms in cases of six A-T base pairs, the minor groove is widened and the major groove is compressed (Fig. 1.10B)(Blackburn 2006, Asagi, Toyama, and Takeuchi 2010).

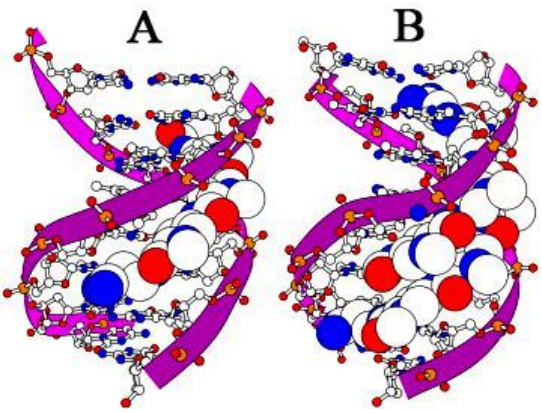


Figure 1.10. **Types of distamycin A-DNA complexes.** (A) 1:1 distamycin A-DNA complex (B) 2:1 distamycin A-DNA complex (Asagi, Toyama, and Takeuchi 2010)

To make possible the recognition of GC sequences, functional groups that make contacts with A-T base pairs should be substituted by other groups, which could recognize GC base pairs. The imidazole containing ligands, namely lexitropsins, which are composed of different aromatic rings connected by peptide bonds, have a high affinity to GC pairs. These types of compounds might be useful in targeting the kinetoplast DNA (kDNA) of parasitic microorganisms since its DNA has a high GC content and inhibition of transcription factors in gene control application (Paul et al. 2015).

1.2.4 Major groove binding

The major groove considerably differs from the minor groove in its characteristics and has different electrostatic potential, hydration, steric effects and hydrogen bonding characteristics (Silverman and Holladay 2014). Preferably, DNA groove binding compounds interact with minor groove and rather small number of compounds manifest ability to bind to the major groove. The first reason probably is the requirement for larger molecules and the second is determined by orientation of nitrogen and oxygen atoms in base pairs to the axis of the helix, thereby contributing to the recognition of major groove mainly by proteins.

The first compound that manifested ability to bind to the major groove was methyl green. Majority of carbohydrates have an ability to interact with major groove. Among them are neocarzinostatin and nogalamycin among and several aminoglycoside conjugates, for example neomycine (Willis and Arya 2006) (Fig.

1.11). Other agents, such as pluramycins, aflatoxins, azinomycins are also considered to be major groove binders (Hamilton and Arya 2012).

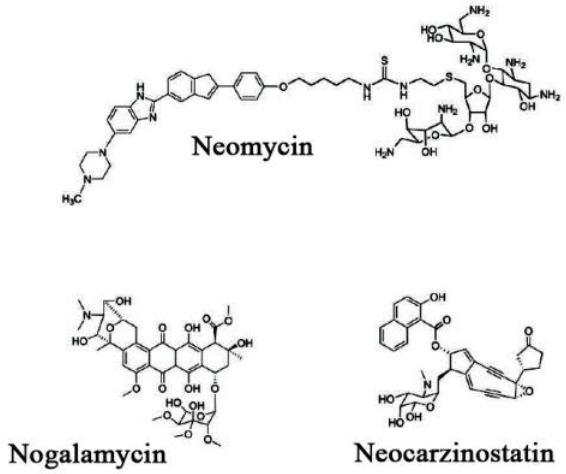


Figure 1.11. **Chemical structures of compounds binding DNA major groove** (Wang et al. 2016).

The most widely known neocarzinostatin chromophore (CNS-chrom) and its derivatives are the antitumor agents and have been used for tumor treatment for decades. It is known that neocarzinostatin molecule forms a complex with protein and causes DNA damage by hydrogen abstraction from DNA strands. Neocarzinostatin-glu and neocarzinostatin –gb are used as DNA-binders, however their binding modes are different. Neocarzinostatin –gb mostly binds to the major groove, meantime neocarzinostatin-glu binds to the minor groove respectively (Gao et al. 2002, Kwon et al. 2003). The ditercalinium also possesses an ability to interact with major groove. However, the number of major groove binders remains small and data on their mechanisms of action are modest.

1.2.5 Intercalation

Intercalation is the most common interaction mode between DNA and ligands, which was first described in 1961 by Leonard Lerman (Lerman 1961). This process involves the insertion of a flat aromatic molecule between DNA base pairs, resulting in DNA unwinding and lengthening, thus interrupting DNA functions (Sheng, Gan, and Huang 2013, K.R et al. 2014). Certain flat aromatic molecules interact with DNA molecule by intercalation and stabilize the duplex without DNA structure disruption. Typically intercalation extends DNA duplex approximately by 3 angstrom (Å) per

bound drug molecule and unwinding degree depends on the site of intercalation and the mechanism of action (K.R et al. 2014). All intercalators possess electron-deficient, an extended planar aromatic ring system and are often referred as chromophores. Traditionally, intercalators contain two or more six-membered aromatic rings and the ligand structure approximately has the same size as DNA base pair. Depending on the number of aromatic parts, intercalative ligands are divided into 2 groups, mono- or bifunctional intercalators. Bifunctional intercalators intercalate in two different ways: intramolecular cross-linking, when ligand intercalates with the same DNA molecule and intermolecular cross-linking when a ligand intercalates with two separate DNA molecules (Fig. 1.12) (Aleksic and Kapetanovic 2014). The intercalative insertion between DNA base pair mainly occurs through hydrogen bonding, hydrophobic and van der Waals forces and the interaction usually occurs with association constant of 10^5-10^{11} M^{-1}. DNA and ligand complex is stabilized by π-π stacking between DNA bases and compound (Blackburn 2006, Sheng, Gan, and Huang 2013, Wang et al. 2016).

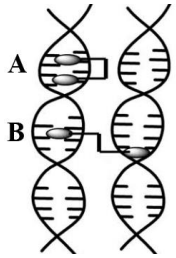


Figure 1.12. **Bifunctional intercalator binding model** (A) Intramolecular cross-linking (B) Intramolecular cross-linking (Aleksic and Kapetanovic 2014)

Previously, only molecules with fused aromatic rings structures were considered to be good intercalators, however molecules with cationic, basic and electrophilic groups also act as intercalators, meantime the ring systems are not always required. (Snyder 2007, Snyder et al. 2005). Intercalators mostly are used as anti-neoplastic and anti-fungal agents and DNA structure studies (Rocha 2015, Biebricher et al. 2015).

The intercalation provokes the base pair separation process, which produces a number of conformational changes in DNA and base pair geometry that is essential for drug accommodation (Blackburn 2006). Depending on the structure of the ligand and the number of involved nucleotides, the separation of bases can vary from 1.8 to 4.5 Å

per intercalation with unwinding angle from 11 to 48˚ (Nakamoto, Tsuboi, and Strahan 2008). The intercalation sites are affected by the ligand size, shape and physicochemical characteristics. Definitely the intercalation sites geometry depends on many factors influencing the intercalation reactivity such as a degree of hydration, ionic strength, pH, cations, structure of DNA and chromosomal proteins (Hendry et al. 2007).

Each ligand prefers a unique binding site. Some prefer to intercalate between 5`-purine-pyrimidine-3` base steps more than to 5`- pyrimidine – purine-3` base step. Meanwhile others preferably choose certain base pair sequences, or do not have preferences at all (Nakamoto, Tsuboi, and Strahan 2008). For instance, antitumor drug adriamycin (also called doxorubicin) prefers the intercalation sites containing GC base pairs, forming specific hydrogen bond between adriamycin and guanine (Yang et al. 2014). One of the most important rule describing intercalative drug accommodation in DNA helix is the nearest neighbour –exclusion principle. In accordance with this principle, every next neighbour intercalation site along the DNA helix remains empty, that is also called negative cooperativity effect. Conformational changes induced by intercalation inhibit binding to the adjacent base pair (Blackburn 2006, Karimi Goftar, Moradi Kor, and Moradi Kor 2014).

The residence time of the drug at DNA site mainly depends on the strength, kinetics and the mode of binding; these are the most important parameters for the biological activity of intercalative compounds (Denny 2002, Rescifina, Zagni, Varrica, Pistara, et al. 2014).

Intercalation is a process which is followed by cytotoxity and most of existing molecules are known as DNA damaging compounds. However there are chemicals with a large range of biological activities which are capable to intercalate, for example hormones, vitamins, antidepressants and antihistamines (Hendry et al. 2007). Some natural compounds, antioxidant polyphenols for example can interact with DNA not only by groove binding but also via intercalation (N'soukpoé-Kossi et al. 2015).

1.2.5.1 Classical intercalation

Classical intercalators contain heteroaromatic moieties with positive charges. Ligands insert their aromatic systems between adjacent GpC DNA base pairs from the top and bottom of the intercalation site (Miskovic et al. 2013, Strekowski and Wilson 2007, Rescifina, Zagni, Varrica, Pistara, et al. 2014). Typical monointercalators are propidium iodide (PI) (Blackburn 2006), acridinium salts including proflavine (also called proflavin and diaminoacridine) which is acridine derivative and ethidium bromide (EtBr) (Nafisi et al. 2007, Nakamoto, Tsuboi, and Strahan 2008) (Fig.1.13). All these representatives contain intercalative chromophore and have cationic charge in neutral pH (Blackburn 2006). Proflavine contains aminogroups, which interact with negative charged phosphate groups. These compounds can easily intercalate between base pairs due to large planer aromatic surface, unwinding DNA molecule, EtBr unwinds for 26 degrees, proflavine - for 17 degrees (Blackburn 2006).

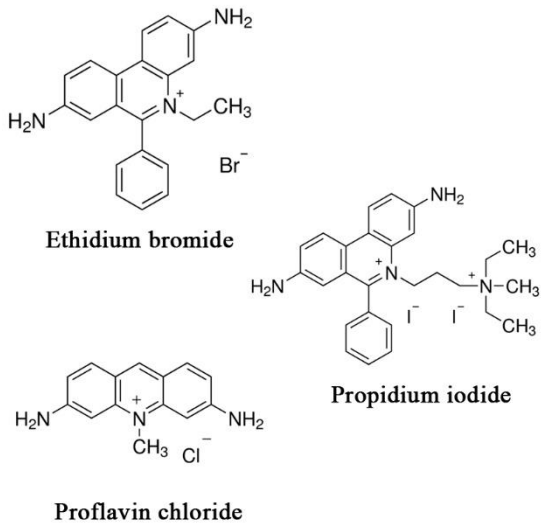


Figure 1.13. **Chemical structures of classical intercalating agents** (Blackburn 2006).

Ethidium bromide (EtBr), acridine orange (AO), acridinium chloride hemi(zinc chloride salt) methylene blue (Nafisi et al. 2007) and diaminobenzidine (Reis, Ramos, and Rocha 2013) are classical intercalators, commonly used as fluorescent stains which are used as a probe for visualizing nucleic acid or proteins. Certain antibiotics and anticancer drugs e.g. actinomycin D and topotecan (Staker et al. 2005) act as classical intercalators as well. Pentapeptide side chains of actinomycin D interact with GC base pairs with 2-amino group of guanine, meantime phenoxazone rings slide into DNA (Fig. 1.14).

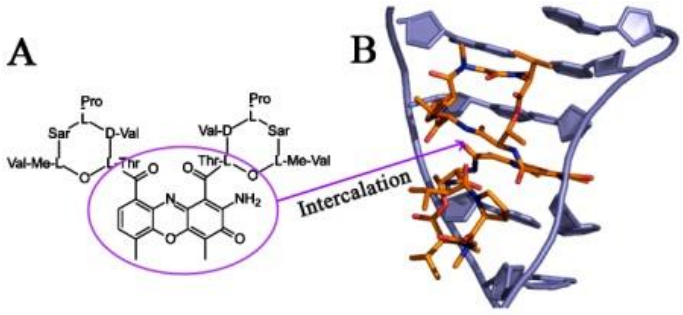


Figure 1.14. **Binding of actinomycin D to DNA** (A) Chemical structure of actinomycin D (B) The intercalation of phenoxazone rings of actinomycin D. Adapted from (Atdbio.com, 2018).

Daunorubicin (daunomycin) and doxorubicin (adriamycin) are well known anthracycline-type antibiotics, which are used to treat a variety of cancers, such as sarcomas, some types of leukemias, myelomas lymphomas, and also cancers of liver, lungs, prostate, ovary, breast and others (Rocha 2015). Daunorubicin and doxorubicin have nearby the same structure (Fig 1.15), both of them consist of a planar ring system, an amino sugar moiety and a fused cyclohexane ring. Daunorubicin and doxorubicine differ only by one extra hydroxyl group, doxorubicin contains it, but daunorubicin does not. In spite of this negligible difference, their biochemical activities are remarkably different.

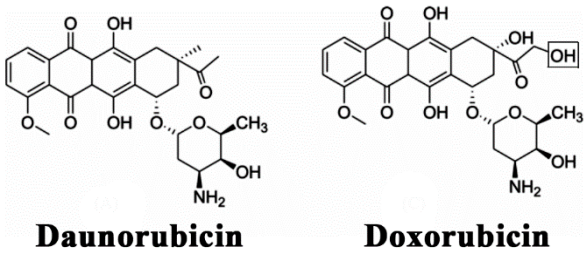


Figure 1.15. **Chemical structures of doxorubicin and daunorubicin** (Sheng, Gan, and Huang 2013).

Daunomycin is most active against leukemias, doxorubicine in turn is more effective against solid tumors (Sheng, Gan, and Huang 2013, K.R. et al. 2014). Perhaps, daunorubicin is the most studied intercalating drug. Initially daunorubicin amino sugar, which is attached to the first anthracycline ring system binds the minor groove, this step activates the rotation of drug, thus resulting in slight deformation of DNA

and allowing two other anthracycline ring insertion between GC base pairs (Fig. 1.16) (Blackburn 2006, Wilhelm et al. 2012).

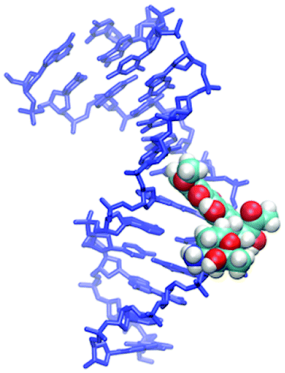


Figure 1.16. **The structure of the daunomycin-DNA complex** (Wilhelm et al. 2012).

Some structural studies showed that daunorubicin preferably inserts between GC base pairs in *triplet sequences* by two adjacent GC and CG followed by AT (Davies et al. 2000). Recently, it was reported what the binding of daunorubucin is stronger with poly(dA-dT)·poly(dA-dT) than for poly(dG-dC)·poly(d(G-dC) and stability of formed daunorubicin-DNA complexes decreased in such order AT-AT>AT-TA>GC-AT>GC-TA>GC-CG>GC-GC. However, daunorubicin affinity increases with GC content for native DNA, but there is no clear explanation for this phenomenon (Barone et al. 2008).

1.2.5.2 Bis-intercalation

Bis-intercalators are large molecules, containing two planar ring systems, which are flexibly linked to each other by linker of varying length (Fig. 1.17). Bis-intercalators are able to bisintercalate into DNA duplex with two planar moieties. Biological activity of intercalator is correlated with binding affinity and dissociation kinetics of the drug. From this point of view bis-intercalators act more effectively, since they have a higher affinity to DNA and dissociate slowlier than single intercalators. Moreover, monointercalators have a smaller binding site; this increases the selectivity of sequence (1 in 3 base pairs). It can be expected that bis-intercalators formed of this kind of monomers have an ability to span at least 6 base pairs.

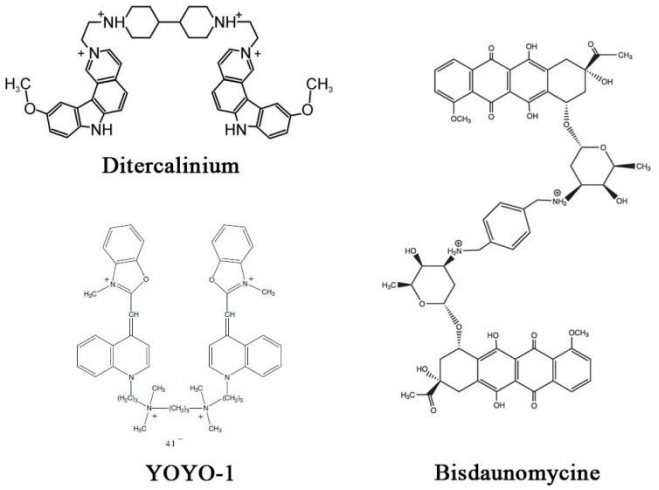


Figure 1.17. **Chemical structure of bis-intercalators** (Berge et al. 2002).

Variable linker lengths allow violating the neighbour-exclusion principle. Planar moieties which are linked by short chains may intercalate at adjacent base pair sites thereby violating the neighbour exclusion rule, whereas bis-intercalators with longer linkers can bind with at least one base separation as required by neighbour exclusion principle (Fig. 1.18) (Blackburn 2006).

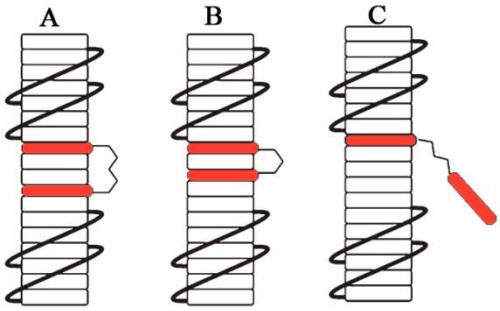


Figure 1.18. **Possible binding modes of bis-intercalators depending on linker length** (Blackburn 2006).

According to the structure, natural bisintercalators which demonstrated antimicrobial, antibiotics and antitumor properties may be divided into 3 classes: (i) cyclic (ii) twofold symmetric bicyclic and (iii) pseudosymmetric bicyclic (Zolova, Mady, and Garneau-Tsodikova 2010).

Natural compounds such as sandramycin , luzopeptins A , quinaldopeptin and quinoxapeptin belong to cyclic bisintercalators. The class of the twofold symmetric bicyclic structures include the thiocoraline , BE-22179, SW-163C, quinomycin B and

C and triostin A. Echinomycin and SW-163D-G are pseudosymmetrical bisintercalators. All these natural bisintercalators contain two planar ring systems and cyclic peptidic backbone, stabilized throught disulfide bridge as in case of triostin A and echinomycin (Aleksic and Kapetanovic 2014). Peptidic backbone of natural bisintercalators plays crucial role in binding specifity and DNA sequence selectivity. For example echinomycin, thiocoraline and SW-163G preferably bind to GC rich sequences, meantime sandramycin, triostin A and luzopeptine A display AT selectivity (Hendry et al. 2007).

Bisdaunomycine, ditercalinium and homodimeric green fluorescent dye YOYO-1 are the most widely used synthetic bisintercalators (Fig. 1.17). Bisdaunomycine (code :WP631), an antitumor and HIV-1 inhibitor is a bisintercalating anthracycline antibiotic (Fig. 1.17), it contains two daunomycine molecules, covalently attached with p-xylene linker to each other, this enables very high binding capacity (Blackburn 2006). Ditercalinium chloride (Fig. 1.17) is a modified dimer of 7H-pyridocarbozole and ellipticine derivative, used as antitumor drug. Its bisintercalation occurs via the major groove and activates abortive DNA repair process leading to the cell death (Sheng, Gan, and Huang 2013).

1.2.5.3 Threading intercalation

Intercalators, which contain bulky substituents groups on opposite edges of planar intercalative moieties, have more complicated binding mechanism than simple intercalators and are called threading intercalations. The threading intercalation is unusual binding mechanism, which comprises the penetration of the planar part of molecule between the base pairs; meanwhile cationic substituent interacts with the major groove and the other bind in the minor groove (Blackburn 2006, Strekowski and Wilson 2007) (Fig 1.19A). When compared to classical intercalators the association and dissociation kinetics of threading molecules are significantly slower but in spite of that they show high binding affinity.

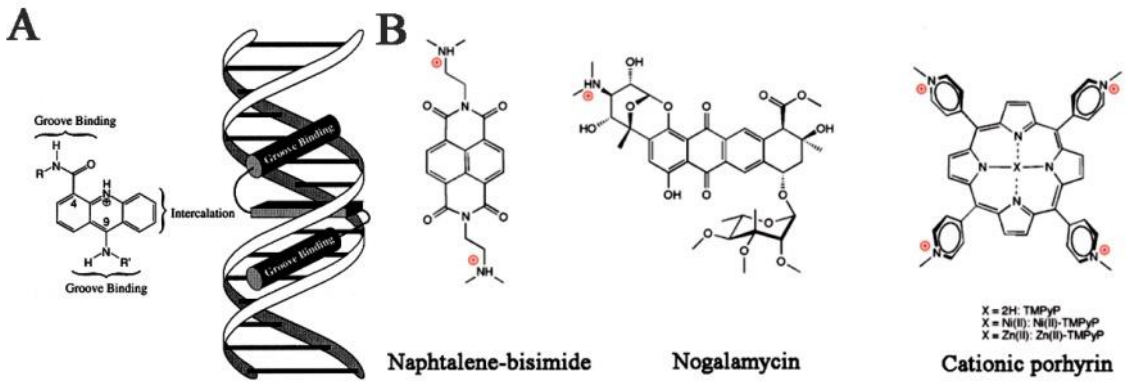


Figure 1.19 **Threading intercalation mode** (A) and structures of threading intercalators (B) Adopted from (Carlson and Beal 2000, Blackburn 2006).

The most studied molecules with threading behavior are nogalamycin, naphthalene-bisamides and cathionic porphyrins (Fig. 1.19B) (Blackburn 2006).

The nogalamycin, one of anthracycline antibiotics possesses an ability to interact with minor and major grooves with high affinity and to thread between phosphodiester backbones. The aglycone moiety of nogalamycin penetrates between DNA base pairs, the uncharged nogalose sugar occupies a position in the minor groove and amino sugar in the major groove (Takagi 2001, Rescifina, Zagni, Varrica, Pistarà, et al. 2014). New threading intercalators were synthesized recently, formulas of these compounds are based on acridine-3- or 4-carboxamide structure (Howell et al. 2010).

1.2.5.4 Multiple binding

Many compounds change their binding mode or have multimodal nature and interact with DNA by different ways simultaneously depending on variety of factors such as base pair sequence, physical internal and external conditions (Rocha 2015).

Ditercalinium and YOYO-1 are bisintercalators, which interact with DNA in two different ways simultaneously through the intercalations and both groove binding. (Sheng, Gan, and Huang 2013, Rocha 2015). Phonoxazone ring of Actinomycin D (ActD) intercalates into DNA at GC-rich sites and two cyclic pentapeptide lactones of the ligand localize in the minor groove (Lo et al. 2013, Chen et al. 2003).

Psoralen is a planar tricyclic drug, which is commonly used for psoriasis treatment, and its interaction mode is mostly dependent on external conditions. Initially this compound intercalates between base pairs but after UVA irradiation it forms covalent

bonds with thymines. However, this kind of treatment can lead to skin cancer (Derheimer et al. 2009).

The concentration of the ligand plays a significant role in binding of drugs. The binding behavior of the fluorescent dye Hoechst 33258 and berenil, which is used to treat trypanosomiasis, are significantly dependent on concentration. Hoechst 33258 is a minor groove binder with AT-rich region selectivity (Fornander et al. 2013). However, there are some reports, which indicate the ability of Hoechst to intercalate at GC-rich regions. Silva et. al drawing on their results proposed that the first binding mode for Hoechst 33258 is intercalation and less dominant is the minor groove interaction (Silva, Ramos, and Rocha 2013). Berenil demonstrates minor groove binding mode but only in low concentrations, if the concentration of berenil is relatively high it acts as intercalator (Wanunu and Tor 2016).

Interestingly, there are some reports about classical intercalator doxorubicin which have an ability to interact with grooves as well, preferably at AT rich regions (Pérez-Arnaiz et al. 2014). DAPI have a high binding capacity with minor groove at AT-rich regions, however it strongly interact with DNA at GC sites via intercalation if the amount of AT bases is pure (Strekowski and Wilson 2007).

1.2.5.5 Nonclassical intercalation

Generally, classical intercalators contain fused polyaromatic ring systems, groove binders also contain aromatic cations, although aromatic systems of groove binders are unfused, that is sufficient for torsial freedom of the ligand, what helps to adopt to twisted DNA shape and to match closely the DNA grooves. Depending on structure, unfused aromatic compounds can bind in two ways: by groove binding or intercalation. It should be mentioned that a majority of unfused ligands intercalate in tandem with groove binding. Intercalators, which contain unfused polyaromatic ring systems and act as groove binders as well, have been named as nonclassical intercalators. The prime examples of this class are a fluorescent stain DAPI and pyrimidine derivatives. Well studied DAPI have a high binding capacity with minor groove at AT-rich regions, however it strongly interacts with DNA at GC sites via intercalation if the amount of AT bases is pure. Moreover, the binding constants of DAPI in this case are comparable with the binding constants of well-known intercalators (Strekowski and Wilson 2007).

1.3 Mechanism of action of antimutagenic compounds

DNA molecule constantly suffers from exogenous agents and ROS formed during cellular metabolism. However, mutations mainly are caused by external factors, including physical and chemical agents called mutagens, which diversity and abundance permanently increase. Mutagenic changes of genetic material that occur in germline cells are the sourse of genomic instability whereas somatic mutations are involved in inception and pathogenesis of various degenerative diseases, including cancer (Sloczynska et al. 2014).

Antimutagenic agents are compounds, which are able to counteract or even remove the effects of potentially harmful agents. Antimutagens include both natural and synthetic compounds, which have ability directly or indirectly inactivate the mutagen, prevent the reaction between mutagen and DNA and enchance the repair kinetics and DNA synthesis (Bhattachar 2011, Sloczynska et al. 2014). According Kada et al. antimutagens are divided in two major groups: desmutagens and bioantimutagens (Kada et al. 1986). Desmutagens are agents which inactivate mutagens (enzymatically or chemically) before they reach DNA, while bioantimutagens, which regarded as true antimutagens,suppress the formation of mutagenic lesions, acting as DNA repair and replication enchancers, thereby decline the frequency of mutation. Antimutagens may be placed in several classes by mechanism of their action. These are compounds with antioxidant activity, with inhibition activity of mutagens, blocking agents and compound which are displaying several modes of action (Sloczynska et al. 2014).

The mechanism of action of many mutagens is the generation of ROS, therefore the effective removal of reactive molecules is a great strategy in process of antimutagenesis. There are wide arrays of antimutagenic compounds which possess free radical scavenging activity, for example carotenoids, flavonoids, phenolic compounds and others (Bhattachar 2011). Several antimutagenic compounds on their own are not antioxidants but can be converted into molecules with antioxidative properties. Such phenomenon was discovered for several amino acid conjugates of curcumin (Parvathy, Negi, and Srinivas 2010).

Another protective mechanism against chemical mutagenesis is the inhibition of mutagen activation. To form active species many promutagens require metabolic activation, which is mediated by enzymes mainly from cytochrome P450 family of

enzymes. For example, the main mechanism of action of isothiocynates is the inhibition of biotransformation of mutagenic compounds via cytochrome P4501A1 and 1A2 activity (Sloczynska et al. 2014).

A number of antimutagenic agents are able directly interact with mutagens before it induces DNA damage. Blocking antimutagens also prevent mutagenic agents reach the target. For example it was suggested that gallic acid bind to the outer membrane transporters leading the blockage of mutagens which are transferred in cytosol (Sloczynska et al. 2014). Antimutagens with multiple mechanism of action provide the protection against a great variety of mutagens and the ability to act in different ways significantly increase the effectiveness of such compounds. For example, phenolics include both intracellular and extracellular mechanisms of action. Firstly its may interfere the biotransformation of mutagens mediated by the cytochrome p450 enzymes and directly interact with active mutagenic metabolites (Marnewick, Gelderblom, and Joubert 2000).

Interestingly, certain compounds which display antimutagenic properties could be promutagenic and carcinogenic as well. Such compounds are termed "Janus mutagens" after the Roman God Janus who had one head with two faces looking in opposite directions. The prime example, which belongs to this group is β-carotene (βCT). βCT is able both scavenge and produce free radicals (Paolini et al. 2003).

1.4 Antimutagens affinity to DNA

Binding of small ligands to DNA mostly is associated with genotoxity and mutagenicity, especially intercalation or groove binding. Over the past several years it was reported that binding of ligands to DNA not always have a negative impact to the functions of DNA. Moreover, it is proved that some compounds have an ability to protect DNA by the interaction via intercalation or groove binding. It was even suggested that nucleophilic bichalcomhenes have an ability bind to DNA, thus protecting genetic material (Marnewick, Gelderblom, and Joubert 2000).

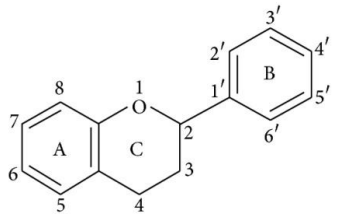


Figure 1.20 **Basic structure of flavonoids** (Kumar and Pandey 2013).

Flavonoids (benzo-y-pyrone derivatives) are a large group of natural polyphenolic compounds which are widely found in plants. Nuclear structure of flavonoids consist of two benzene rings (Fig. 1.20A,B), which are linked by heterocyclic pyrane ring (Fig. 1.20C). Flavonoids can be divided in different classes, such as flavonols, where kaempferol, quercetin and myricetin are the best known representatives; flavones comprising luteolin, apegenin and chrysin; flavonones which include flavonone and narengenine and others, for example, anthocyanins and isoflavones (Kumar and Pandey 2013). Flavonoids have various pharmaceutical and biochemical activities including anticancer, antidiabetic, neuroprotective, antibacterial and antioxidant activities. Flavonoids are well known mainly as strong antioxidants and free radicals scavengers (Bukhari et al. 2009, Kamalakkannan and Prince 2006). Moreover many flavonoids provide greater antioxidant capacity than vitamins C and E (Prior and Cao 2000). Flavonoids reduce the extent of oxidative stress by scavenging of ROS, chelation of transition metal ions Fe II and Fe III, preventing Fenton reaction, activating antioxidant enzymes (Nijveldt et al. 2001), reducing α-tocopheryl radicals (Hirano et al. 2001), inhibiting oxidases, such as xanthine oxidase and protein kinase C (Akhlaghi and Bandy 2009), mitigating oxidative stress caused by nitric oxide by the NOS inhibition (Matsuda et al. 2003) and scavenging of peroxynitrite (Heijnen et al. 2001). The biochemical activity of flavonoids depends on their structural class, substitutions and conjugations, degree of hydroxylation and polymerization (Heim, Tagliaferro, and Bobilya 2002). Interestingly, some flavonoids with high antioxidant and antimutagenic activity have an ability to interact directly with DNA molecule via both intercalation and groove binding (Kanakis et al. 2005). It may be assumed that it is one of the way to protect DNA from other mutagens.

Resveratrol (3,4,5`-trihydroxystilbene), genistein (4`,5,7-trihydroxy isoflavone) and curcumin (diferuloyl methane) are the bright representatives of antioxidant

polyphenols which have an ability to interact with DNA molecule (Fig. 1.21.) All of them manifested antitumor potential and protected against cardiovascular diseases. Resveratrol is a phytoalexin that is found in berries, grapes, wine, and play very important role in cancer prevention. Particularly resveratrol is able to delay cell cycle progression and induce apoptosis in several cancer cell lines (Leone et al. 2010). Genistein, mostly found in soybeans, demonstrated anticancer activity against breast cancer (Xie et al. 2014). Curcumin is a yellow pigment from the rhizome of *Curcuma longa* that exhibits anticancer activity and produces anti-infectious and anti-inflammatory effects (Sikora et al. 2006). Using multiple spectroscopic studies and docking analysis the binding ability of resveratrol, genistein and curcumin were investigated. It was reported that resveratrol and genistein bind both via intercalation and groove binding, meantime curcumin form only groove binding adducts. Resveratrol is the stronger binder comparably with genistein and curcumin. Moreover binding of that compounds did not alter B-DNA conformation (N'soukpoé-Kossi et al. 2015).

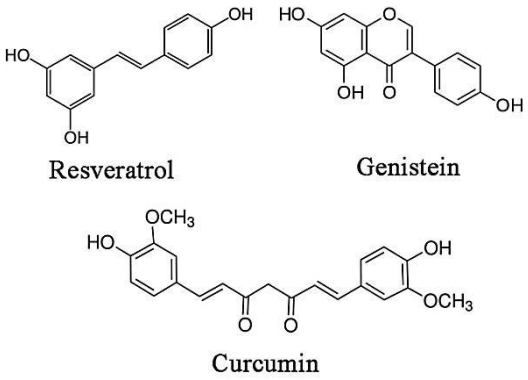


Figure 1.21. **DNA binding flavonoids**

Morin, rutin and quercetin as well form the intercalative complexes with DNA. Quercetin showed the greater binding capability with DNA (Janjua et al. 2009). 3-Hydroflavone (3HF), which exhibits very strong antioxidant activity has very similar structure with quercetin differing with the number of hydroxyl functional group, but unlike quercetin 3HF acts as a groove binder (Jana et al. 2012).

Saffron and their derivatives crocin, crocetin, safranal and dimethylcrocetin from *Crocus sativus* are considered as very important bioactive compounds with a high anticancer, antioxidant, antidiabetic and anti-inflammatory potential and have

numerous applications of their pharmacological potential (Bukhari, Manzoor, and Dhar 2018). Nevertheless, intercalative and external binding modes of saffron compounds were recently observed (Kanakis et al. 2009).

Quinolines and their derivatives have a great biological importance exhibiting antibacterial, antimalarial, anticancer, antiviral, hypoglycaemic (Sri Ramya et al. 2018) antioxidative effects (Puskullu, Betul, and Sibel 2013) and others. The synthesized novel chloroquinoline derivatives 1-(2-chloro-4-phenylquinolin-3-yl)ethanone (CPQE), 1-(2,6dichloro-4-phenylquinolin-3-yl)ethanone (DCPQE), methyl 2,6-dichloro-4-phenylquinoline-3-carboxylate (MDCPQC), methyl 2-chloro-4-methylquinoline-3-carboxylate (MCMQC) are promising anti-diabetic and antioxidant compounds, which effectively reduce the high glucose level and possess antioxidant activity. All these novel agents showed a good binding affinity to DNA (Murugavel et al. 2017).

The anti-diabetic drug metformin (N,N-dimethylbiguanide) is a widely used compound to treat hyperglycaemia in patients with type 2 diabetes mellitus. However, metformin exhibits an extraordinary diversity of biological activities. Metformin elevates endogenous reactive oxygen species and reduces oxidative damage of DNA (Algire et al. 2012). It was proved that metformin possesses significant anticancer effect against various types of cancer (Kasznicki, Sliwinska, and Drzewoski 2014). Metformin protects against cardiovascular diseases and slows down ageing processes (Bridgeman et al. 2018) and has beneficial effects in various neurological disorders (Jang and Park 2018). The ability of metformin to interact with DNA by minor and major groove binding, preferably with AT rich regions was recently reported (Mondal et al. 2018).

1.5 1,4-dihydropyridine derivatives (1,4-DHP)

Arthur Hantzsch synthetized the nucleus of 1,4-DHPs more than one century ago (Fig. 1.22) (Hantzsch 1881). However, the biological activities of these compounds were discovered 60 years later. The derivatives of 1,4-DHPs are small, nitrogen containing heterocyclic rings, saturated at 1^{st} and 4^{th} positions (Ioan et al. 2011). 1,4-DHPs represent a class of the most successful small molecules which are widely used in medicine and pharmacy. The derivatives of 1,4-DHP may bear various substituents at positions 1,4-, 2,6 and 3,5. Mostly 1,4-DHPs act as calcium channel blocking agents,

these are the most frequently used compounds for the treatment of cardiovascular diseases (Triggle 2007), however some of them behave as activators (Ioan et al. 2011, Edraki et al. 2009). Heterocyclic ring is an important feature for various pharmacological properties of DHPs such as antianginal, antihypertensive, antitumor, anti-inflammatory, antitubercular and antitrombothic (Razzaghi-Asl, Miri, and Firuzi 2016, Khedkar and Auti 2013, Swarnalatha et al. 2011). Moreover 1,4-DHPs demonstated many more biological activities such as neuroprotection activity (León et al. 2008), HIV-1 protease inhibition (Hilgeroth and Lilie 2003), memory enchancing effect (Klusa 2006), mitochondria – protecting activity (Fernandes et al. 2003), anti-oxidant properties (Milkovic et al. 2018, Ryabokon et al. 2005), antimutagenic activity (Goncharova et al. 2001, Vartanian et al. 2004), DNA repair stimulation activity (Ryabokon et al. 2005) and anti-diabetic activity (Briede et al. 2008) and many other properties.

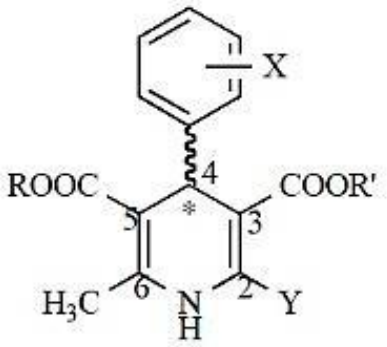


Figure 1.22. **Structure for the most common 1,4-dihydropiridine** (Carosati et al. 2012).

1,4-DHPs can be divided into 4 generations according to their pharmokinetic and pharmacologic characteristics (Wang, Iadecola, and Wang 2017). The first generation drugs, nifedipine for example, produce effect with rapid onset and short duration time, with side effects, mainly baroreflex activation (Coca et al. 2013); the second generation drugs are characterized as slow release drugs, slow –release nifedipine or newly syntetized isradipine, nicardipine and felodipine are slow-acting drugs with better control of therapeutic effect and reduced baroflex activation (Wang, Iadecola, and Wang 2017); the third generation - amplodipine, lercanidipine and barnidipine (Wang et al. 2011, Liau 2005, Ozawa, Hayashi, and Kobori 2006) are long-acting drugs and exhibit more stable pharmakinetics. These drugs are less cardio-selective thereby well tolerated in patients with heart failure, especially in patients with chronic

kidney disease (Malhotra and Plosker 2001); the fourth generation are L/N type calcium channel blockers cilnidipine and lercanidipine with a high degree of therapeutic comfort and stable activity. This kind of drugs is used for treatment of myocardial ischemia and heart failure (Chandra and Ramesh 2013).

1.5.1 Atypical 1,4 –DHPs

DHPs with added "free" amino acid residue (taurine, glutamate, alanine or GABA) to the ring at position 4, instead of phenyl group, which is typical for classical DHPs, at low doses regulate and normalize neurotransmitter activities of brain and behavior. Compounds were named depending on amino acid moieties, for instance glutapyrone, tauropyrone, gammapyrone and alapyrone (Fig. 1.27) (Klusa 2016)

Glutamate containing glutapyrone is an atypical representative of DHPs, due to the lack of calcium antagonism properties, which are typical of the classical DHPs. Glutapyrone is the most studied amino-acid containing DHP which possess a variety of beneficial biological actions such as anticonvulsive activity , stress-protective effect (Tarasenko, Neporada, and Klusha 2002), antimutagenic and DNA repair enhancement activity , antioxidant, cardio and gastric tissues protective properties (Tarasenko, Neporada, and Klusha 2002). Moreover glutapyrone prevents mitochondrial myopathies in skeletal muscles, effectively reduce neuro- and cardiotoxicity, prevent inflammation and apoptosis caused by azidothymidine, which is a widely used compound in treatment of immune deficiency syndrome and considerably improved memory of previously alcoholized rats in gestation period (Pupure et al. 2008).

Tauropyrone , a taurine containing 1,4 –DHP are less studied representatives, however tauropyrone acts as antineurotoxic and memory improving agent (Klusa et al. 2006, Della Corte et al. 2002) and prevents the aggregation of human blood cells (Velena et al. 2016). Tauropyrone diminish inflammatory processes in 6-OH-dopamine model of Parkinson`s disease in rats and in migloglial cells *in vitro* (Ward et al. 2006). Gammapyrone acts as neuroprotector as well and protects mitochondria against oxidative stress (Fernandes et al. 2003).

Cerebrocrast (Fig. 1.27) a difluoromethoxyphenyl group containing DHP is also one of the mostly studied representative of DHP derivatives among compounds with

cardioprotective effects. Cerebrocrast acts as inflammatory and neuroprotective agents. For example, cerebrocrast inhibited secretion of interleukines IL-1β and IL-6 and neurotoxic products by cells in human monocyte (THP-1) cell line in a model of rat paw edema induced by carragenan (Klegeris et al. 2002) Along with glutapyrone, mitochondria protecting effects of cerebrocrast also were reported in the presence of azidothymidine (Velena et al. 1997). Moreover cerebrocrast demonstarted anti-diabetic activity by the significant reduction of glucose level in blood of animals with diabetes and protected pancreatic cells from the streptozotocin (STZ) induced damage (Briede et al. 2004, Briede et al. 2007). Another data have not indicated that cerebrocrast has hypoglycaemic effect, but it down-regulated nitric oxide (NO) levels in kidneys, liver and blood (Leonova et al. 2016). Cerebrocrast has a high antioxidative capacity, it reduces the death of cerebellar granule cells, induced by 1-methyl-4-phenylpyridinium (MPP+) which cause dramatic oxidative stress and effectively decrease generation of ROS (Klimaviciusa et al. 2007).

Recently it was reported that etaftorone and fenoftorone another representatives of atypical DHPs possess the anti-diabetic properties (Fig. 1.27). These effectivelly normalized NO production in kidneys, blood and muscles in the diabetic rats. Etaftorone was more active than cerebrocrast and fenaftorone. Moreover etaftorone down-regulated inducible nitric oxide synthase (iNOS) and xanthine oxidase (XOR) enzyme expression, regulated eNOS enzyme in kidneys and decreased morphological changes in kidneys of diabetic rats (Leonova et al. 2016).

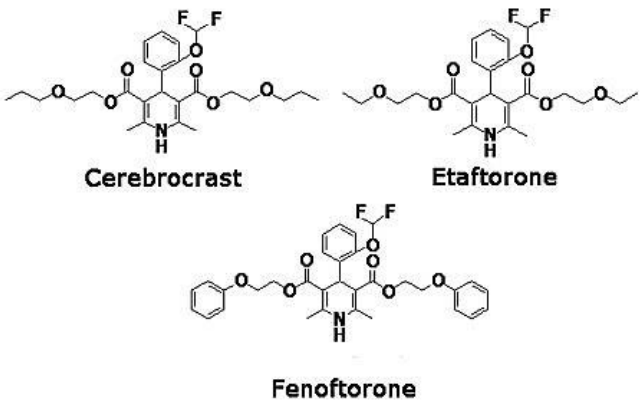


Figure 1.27. **General structure of atypical 1,4-DHP derivatives.**

1.5.2 Antioxidant activity of 1,4-DHPs

Several representatives of 1,4-DHPs and their metabolites demonstrate a high AOA and antiradical activity (ARA) and that is why could be considered as a strong bioprotectors against oxidative stress and associated diseases (Godfraind 2005).

1,4-DHPs are analogous of 1,4-dihydronicotinamide which is an active part of nicotinamide adenine dinucleotide phosphate (NADPH) coenzyme, and is involved in a enzymatic redox reactions of hydrogen (H^+) and electron transfer (Duburs et al. 2008).

2,6-dimethyl-3,5-diethoxycarbonyl-1,4-dihydropiridine, Hantzsch ester (HEH), namely diludin, was the first compound which demonstrated the AOA. Diludin and its analogues are 4-unsubtituted 1,4-DHPs which were created by Latvian scientists. Depending on molecular structure, 1,4-DHPs have a considerable hydrogen donor ability with a possibility to inhibit directly reactions of free radicals. (Velena et al. 2016). N-heteroaromatic and partly hydrogenated nucleous of 1,4-DHP and its fragments contain NH group or C-4-H atom which serve as hydrogen donors and C=O group and O atom in position 3 and 5 (ester side groups) and 2 and 6 (alkyl side groups) as hydrogen acceptors (Tikhonov and Zhorov 2009). The presence of hydrogen atoms in position 1 and 4 significantly increases antioxidative properties of these compounds and the substitutions in position 4 strongly diminish the AOA (Carosati et al. 2012). 3,5-dicarbonyl-1,4-dihydropiridine systems are considered as C-H antioxidants. The transfer of hydrogen of 3,5-dicarbonyl-1,4-dihydropiridine results in the formation of corresponding pyridine (Augustyniak et al. 2010) (Fig. 1.28).

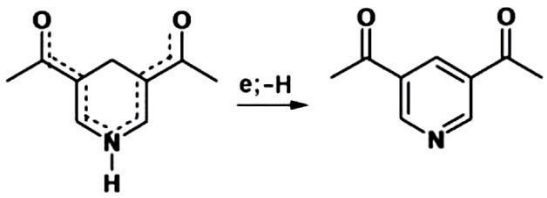


Figure 1.28. **Reaction of 1,4-dihydropiridines leading to the formation of pyridine derivatives** (Augustyniak et al. 2010).

DHPs react with different types of free radicals, alkyl radicals, and oxygen and nitrogen free radicals and with stable free radicals (DPPH a.o.). Moreover, several

DHPs have the ability to quench singlet oxygen by electro donation (Lopez-Alarcon et al. 2004, Buettner and Mason 2003, Tarpey and Fridovich 2001, Yanez et al. 2004).

It also was demonstrated that 1,4-DHPs have an ability to quench peroxynitrite ($ONOO^-$) by the direct reaction with $ONOO^-$ (Fig. 1.29) (Lopez-Alarcon et al. 2004).

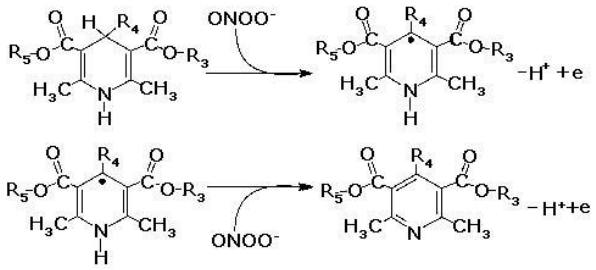


Figure 1.29. **Reaction between 1,4-DHP and peroxynitrite.** Adapted from (Lopez-Alarcon et al. 2004).

1.5.3 AV-153-Na

Sodium 3,5-bis-ethoxycarbonyl-2,6,dimethyl-1,4-dihydropyridine-4-carboxylate (AV-153), synthesized in the Latvian Institute of Organic Synthesis (Duburs and Uldrikis, 1969). AV-153-Na is an analogue of the active center of the reduced form of NADH or its phosphate NADPH (Fig 1.30)

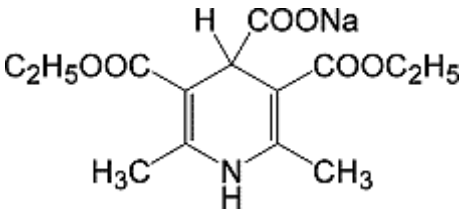


Figure 1.30. **Chemical structure of AV-153-Na**

AV-153-Na is considered as "unusual" representative of 1,4-DHP, without or really weak blocking activity of Ca^{2+} channels. AV-153-Na possesses antimutagenic, antioxidant and DNA repair enhancing activity.

AV-153-Na protects against chemical mutagenesis and reduce the frequency of chromosome breakage and point mutation induced by alkylation in *Drosophila melongaster* (Goncharova and Kuzhir 1989), chromosomal aberrations induced by radiation and other cytogenic end-points in fish (Goncharova 2000) and micronuclei induced by alkylation in cells of bone-marrow in mouse (Goncharova et al. 2001).

AV-153-Na significantly reduced mutation frequency and DNA damage in hypoxanthine-guanine phosphoribosyl transferase (HGPRT) lokus in hamster ovary cell and decreased the level of 8-oxo-7,8-dihydro-2`-deoxyguanosine (8-oxodG) level and mutation frequency after X-irradiation about 40% (Wojewódzka et al. 2009).

It is proved that AV-153-Na effectively decreases DNA oxidative damage produced by hydrogen peroxide and stimulate DNA repair kinetic after oxidative stress in peripheral lymphocytes (Ryabokon et al. 2005, Ryabokon et al. 2008). Osina and co-aughtors have found that AV-153-Na up-regulates expression of the proteasomal *Psma6* gene, polymorphisms of this gene are is associated with *diabetes mellitus* (DM) and cardiovascular diseases (Osina, Rostoka, Sokolovska, et al. 2016). Another study of the same authors reported that AV-153-Na down-regulates the level of poly [ADP-ribose] polymerase (PARP1) and iNOS expression and increases the expression of eNOS in diabetic animals that could be prospective for treatment of DM complications (Osina, Rostoka, Isajevs, et al. 2016).

1.6 Oxidative stress and DNA lesions

1.6.1 Reactive oxygen species and reactive nitrogen species

DNA lesions induced by oxidation have been investigated for decades due to pivotal role of oxidatively damaged DNA in mutagenesis, carcinogenesis and generative diseases of aging Oxidative stress is closely associated with a molecular pathogenesis of development and progression of various disorders and diseases (Evans, Dizdaroglu, and Cooke 2004).

ROS is a group of highly reactive compounds comprising free radicals (molecules with unpaired electron) such as superoxide anion radical (O_2^-) and hydroxyl radical (·OH) and non-radical such as hydrogen peroxide (H_2O_2). Moreover ROS include also diverse peroxides, which are produced by the reaction of ·OH and $O2^-$ with organic compounds, such as peroxides of proteins, lipid peroxides (LOO·) and nucleic acids. Also there are various reactive species (RS) of nitrogen, such as nitric oxide (NO) and $ONOO^-$ (Lushchak 2014). $ONOO^-$ which is nitrating agent and very strong oxidant is produced by the reaction with $O_2^{\cdot -}$ with NO (Beckman et al. 1990, Radi et al. 2001) (Equation 1). $ONOO^-$ is equilibrium with the corresponding peroxynitrous acid (ONOOH) (Equation 2). However the stability and reactivity of $ONOO^-$ and ONOOH is quite different. The decomposition of ONOOH leads to ·OH and $\cdot NO_2$ production.

The reaction of $ONOO^-$ with carbon dioxide form very reactive nitrosoperoxycarbonate ($ONOOCO_2^-$)(Pryor, Jin, and Squadrito 1994). At physiological pHs $ONOOCO_2^-$ and $ONOO^-$ rapidly decompose forming ·OH and CO_3^-(Radi 2013).

$O_2^{\cdot-} + \cdot NO \rightarrow ONOO^-$

(Eq.1)

$ONOO^- + H^+ \leftrightarrow ONOOH$

(Eq. 2)

ROS and free radicals, most notably ·OH, instantly react with macromolecules including DNA and negatively impact their structure. Any modification in DNA structure caused by oxidation induce abasic sites (AS), base modification and strand breaks thereby altering coding properties and interfere with cell metabolism (Krebs and Goldstein 2014).

1.6.2 Mechanism of oxidative damage of DNA

Free radicals, mostly ·OH interacts with DNA by addition to double bonds, where H atom is abstracted from the methyl group of thymine (Fig 1.32) and from each of the five carbon atoms of 2`-deoxyribose. The reaction of addition reverently occurs at sites of DNA bases with the highest electron density. The addition reactions produce OH adducts of DNA bases. Further reactions of the C- or N- centred radicals of DNA bases and C- centred radical of sugar are formed from abstraction. The presence of oxygen leads to peroxyl radicals formation by the addition to OH-adducts radicals and C-centred radicals. Hydrated electrons produce electron adducts by the addition to the double bonds of pyrimidine. H atoms also have the ability to react with pyrimidines. OH –adduct radicals of pyrimidines and allyl radicals can be further oxidized or reduced, depending on their redox properties, resulting a variety of products. Such modification of sugar and base radicals leads to a modified sugars and bases formation, base free sites, crosslinks and strand breaks (Dizdaroglu and Jaruga 2012b).

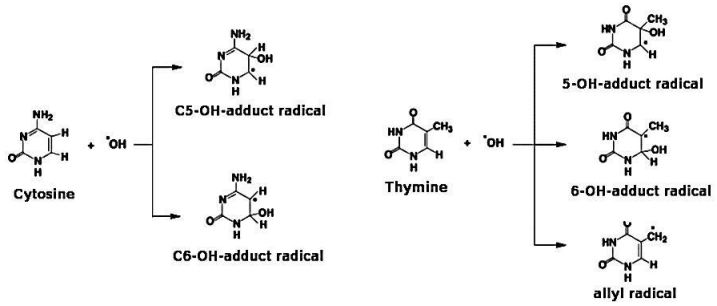


Figure 1.32. **Reactions of pyrimidines with hydroxyl radical** (Evans, Dizdaroglu, and Cooke 2004).

The majority of DNA lesions induced through oxidation are single lesions; however there is possibility of multiple lesions or tandem lesions then two lesions are formed on adjacent nucleotides. Tandem lesions mostly are formed by the interaction of ˙OH with DNA moiety and radiation insult (Dizdaroglu and Jaruga 2012a).

1.6.2.1 Oxidative damage of DNA bases

Among the four DNA bases, guanine (G) has the lowest reduction potential, thereby it is preferentially oxidized base . Hydroxyl radical adds to guanine at C4-,C5- and C8-positions, thereby forming OH-adduct radicals of guanine and sometimes at C2-, but less frequently (Fig. 1.33) (von Sonntag 2006). C4-OH and C8-OH-adduct radicals are forming an extent of 65-70% and 17%, C5-OH is forming less than 10% (Candeias and Steenken 2000, Evans, Dizdaroglu, and Cooke 2004).

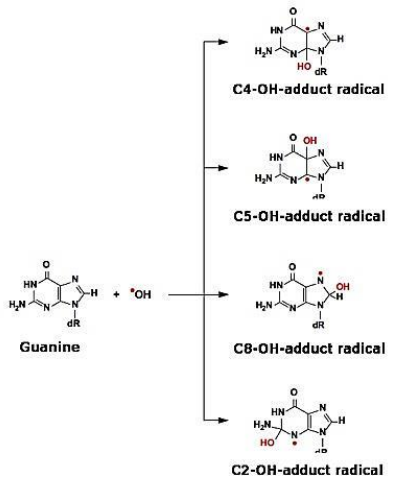


Figure 1.33. **Reaction of guanine with hydroxyl radical** (Dizdaroglu and Jaruga 2012a).

The C4-OH-adduct radicals of guanine are vulnerable to oxidation while C5-OH and C8-OH are being primarily reducing. However OH-adduct radicals of purines represent 'redox ambivalence' and might be oxidizing and reducing because they could exist in different mesomeric forms (Candeias and Steenken 2000). After formation of the C4-OH- and C5-OH adducts, considerable conformational changes occur within molecule. The elimination of water of C4-OH and C5-OH adduct radicals give a rise of neutral G radicals [G (-H·] which after that are protonated generating the guanine radical cations ($G^{\cdot+}$). G (-H·) and $G^{\cdot+}$ are strong oxidants. G (-H·) and can be reconstituted by reduction. $G^{\cdot+}$ upon water addition gives a rise of C8-OH-adduct radical. In contrast to $G^{\cdot+}$, G ($^{-}$H·) does not generate the 8-oxoguanine (8oxoG). However, it reacts with 2'-deoxyribose by an abstraction of H· at 2'-deoxyribose that's lead to formation of C-centred radicals of 2´deoxyribose lesion and give a rise of strand breaks. C8-OH-adduct radical reacts with oxygen and forming 8-oxoG. This reaction competes with the opening of imidazole ring by the scission of C8-N9 bond yielding 2,6-diamino-4-hydroxy- 5-formamidopyrimidine (FapyGua) (Fig. 1.34) (Dizdaroglu and Jaruga 2012b, Evans, Dizdaroglu, and Cooke 2004).

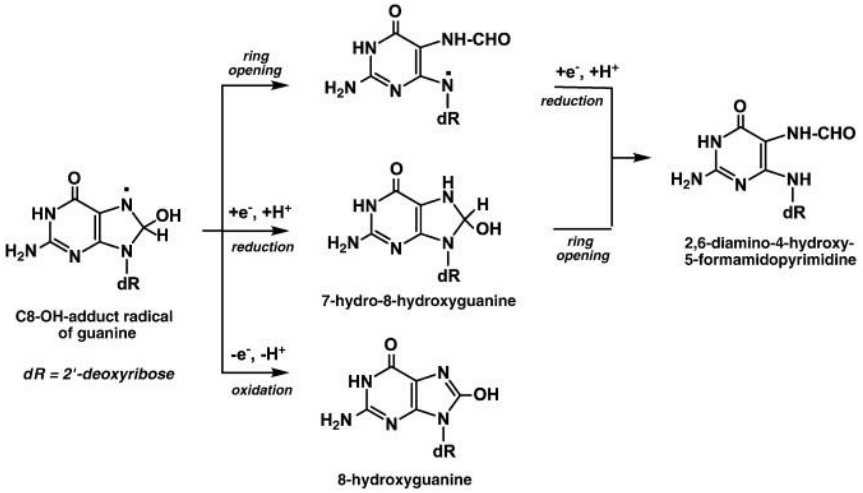


Figure 1.34. **Formation of Fapy from the reaction of 8-OH-adduct radical** (Evans, Dizdaroglu, and Cooke 2004).

Nowadays 8-oxoG recognized as one of the most important marker of oxidatively damaged DNA (Kasai 2016, Suzuki and Kamiya 2017) and this lesion has a high mutagenecity in mammalian cells (Suzuki and Kamiya 2017). It was revealed thet 8-oxoG may adopt two *anti* and *syn* conformations. During DNA replication guanine oxidation products cause G:C-C:G and G:C-T:A transversions. The *anti* conformation

of 8-oxoG forms Warson-Crick base pair with cytosine while the *syn* conformation uses the Hoogsteen edge to form base pair with adenine These mutations were found in many important genes, for example in the *p53* tumor supressor gene and at codons 12 and 13 of the *K-ras* gene (Whitaker et al. 2017a). 2,5-diamino-4H-Imidazol-4-one (Iz) and 2,24-Triamino-5(2H)-oxalozone (Oz) are another oxidative products of both guanine and 8-oxoG .It was revealed that Iz and Oz induce G:C-C:G transitions as well (Kino et al. 2009, Suzuki et al. 2012).

Hydroxyl radicals react with thymine and cytosine at positions C5 and C6, generating C5-OH and C6-OH adducts. In the case with cytosine approximately 87% of OH· adds to the C5 and 10% to the C6. In case with thymine 60% of the OH adds to C5 and 30% to C6 (Dizdaroglu and Jaruga 2012a) The oxidation of C5-OH-adduct radicals of thymine and cytosine followed by hydration and deprotonation result the formation of thymine glycol (Tg) and cytosine glycol (Cg) (Fig. 1.35) The abstraction of H· leads to allyl radical formation of thymine. The oxidation of the allyl radical of thymine results the 5-hydroxymethyuracil (5-OHMeUra). The reaction of allyl radical with oxygen increases the production of 5-OHMeUra and 5-formauracil (5-FoUra) (Valko et al. 2004) .

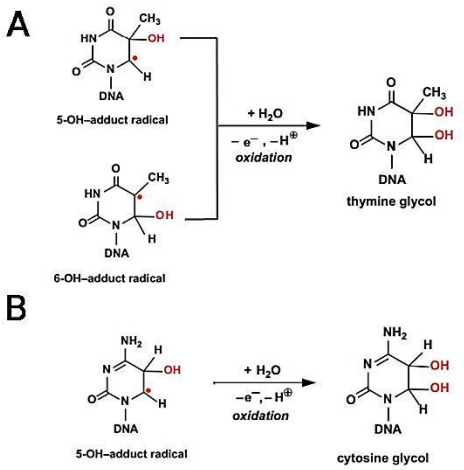


Figure 1.35. **The mechanism of formation of thymine glycol (A) and cytosine glycol (B).** Adopted from (Dizdaroglu and Jaruga 2012a).

In case with adenine (A) OH· attacts at C4 and C8 position, forming C4-OH and C8-OH-adduct radicals at 50% and 37% respectively. The frequency of OH· addition to C5 is approxymately 5%. The addition of OH· to C2 as suggested also take place but not more than 2% . C2-OH-adduct radical by the reaction with oxygen promote a rise

of 2-hydroxyadenine (2-OH-Ade) by one electron oxidation. C4-OH radical undergoes dehidratation yielding strongly oxidizing A (-H·). Similarly to G (-H·) it become protonated and give $A^{\cdot+}$ which upon hydration produce C8-OH-adduct radical The oxidation of the C8-OH-adduct radical by one electron generates 8-hydroxyadenine (8-OH-Ade). Competing with oxidation reaction, this radical undergoes ring opening, followed by one-electron reduction , 4,6-diamino-5-formamidopyrimidine (FapyA) is produced (Dizdaroglu and Jaruga 2012a).

Peroxynitrite-induced DNA oxidative lesions are associated with oxidation of guanine resulting products 8-oxoG and 2,5-Diamino-4H-imidazol-4-one (Iz). The reactivity of peroxynitrite is highly pH dependent. It was found that $ONOO^-$ induce the oxidation of adenine mainly in position C8, producing 8-oxodeoxyadenosine (8-oxodA) as well (Sodum and Fiala 2001).

1.6.2.2 Damage to the sugar moiety of DNA

Hydroxyl radicals attact sugar moiety of DNA by the abstraction of H atom from each carbon atom of the 2'deoxyribose, in order H5′>H4′>H3′≈H2′≈H1′, generating five C-centered radicals which undergo further reactions, giving a rise of a variety of the sugar modifications . Damage of 2'-deoxyribose in DNA leads to strand breaks, abasic sites and therefore release of unaltered DNA bases. Some modified sugars as a free products are released from DNA, while others remain within DNA or constitute end groups of broken DNA strands (Evans, Dizdaroglu, and Cooke 2004). Generally the amount of OH· attack on 2'-deoxyribose is less than 20% (von Sonntag 2006). However the extent of DNA strand breakage is greater due to radical transfer from a base to sugar (Evans, Dizdaroglu, and Cooke 2004). The most understood mechanism that leads to DNA breakage and products is the reactions of C4'-radical in the absence of oxygen. This radical is an alkoxyalkyl radical with a phosphate group in the β position. Thereby it readily loses the phosphatgroup on both side of the DNA chain and forms two different radical cations. Cleavage of phosphate group at C3 and C5 leads to formation of two radical cations. These radicals hydration and reduction followed by release of unaltered base results 2,3-dideoxypentos-4-ulose and 2,5-dideoxypentos-4-ulose as ends groups in broken DNA chains . C4'-radical undergoes oxidation and hydration (addition of OH^-) followed by reduction and elimination of the unaltered base yields a 2-deoxypentos-4ulose moiety within DNA. C4 radical

rapidly reacts with oxygen leading to a peroxyl radical formation which undergoes β-fragmentation resulting the formation of glycolic acid residue as a 3′-end group . C2 radical induce the formation erythrose within DNA, which is a fragmented sugar ring product . C1-peroxil radical leads to erythrose formation as well. C5-peroxyl radical yields 2-deoxytetradialdose generation as an end group of a broken DNA chain. Moreover C5 radical can be envisioned for the generation of the 5′-aldehyde (Dizdaroglu and Jaruga 2012a).

1.7 Repair of DNA oxidative damage

Base lesions induced by ROS are repaired by two types of activity. Base-excision repair (BER) is the main mechanism and repair majority of oxidized DNA damage. Specific DNA glycosylases, AP-endonucleases, DNA polymerases and DNA ligases are essentially basic components of BER (Whitaker et al. 2017b). Nucleotide excision repair (NER) is a second more complex process, which comprises a multiple lesion recognition, insicion proteins, DNA re-synthesis and ligation (Melis, van Steeg, and Luijten 2013).

8-oxoG, formamidopyrimidines (FapyG and FapyA), Tg and other base modifications are removed using BER pathway. Innitially damaged base is identificatified and removed by specified glycosylases, such as 8-oxoguanine DNA glycosylase (OGG1), endonuclease III-like 1 (NTH1), endonuclease VIII like (NEIL)1, NEIL2, NEIL3, and mutY homologue (MYH) which is specially designed to remove mismathes 8-oxoG·A and 8-oxoG:G incorporated during DNA replication (Hegde, Hazra, and Mitra 2008, Evans, Dizdaroglu, and Cooke 2004).

The oxidation of DNA bases induces SSBs with dirty ends comprising sugar fragments or 3′phosphates. These blocking groups include 3′phosphoglycolate, 3′phosphate or 3′phosphoglycoaldehyde. In the initiation steps of BER, damage specific mono –or bi-functional DNA glycosylases (DGs) identifies and excises the modified base (Whitaker et al. 2017b).

GGs utilize both monofunctional and bifunctional mechanism, depending on bonds which are cleaved at the time of removal of damaged nucleobase. Monofunctional DGs cleave only the N-glycosil bond but are not able to cleave a phosphodiester bond. Uracil and alkylated bases are recognized and removed by monofunctional DGs

trough cleavage of the N-glycosilic bonds between the target base and the deoxyribose, leaving apurinic/apyrimidinic (AP) sites. Bifunctional DGs are divided into β eliminators which cleave the phosphate backbone on the 3′side and β,δ eliminators which cleave both 5′ and 3′ of abasic sites forming a gap (Whitaker et al. 2017b). NEIL1 NEIL2 and NEIL3 glycosilases are highly relevant to oxidative stress with target of 8-oxoG, FapyG and FapyA. These DGs carry out β- and δ-elimination reactions generating SSB with 3′P and 5′P termini followed by the removal of 3′P group by polynucleotide kinase (PNK), leaving a hydroxyl at the 3′terminus.(Hegde, Hazra, and Mitra 2008)

OGG1, NTH1 glycosilases possess β elimination mechanism and induce SSBs leaving 3′phospho α,β unsaturated aldehyde (3′PUA) and 5′P of the termini of the repair gap. AP sites induced β elimination reaction are processed by human AP endonuclease 1 (APE1) leaving behind the SSB with 3′hydroxyl and 5′deoxyribosephosphate (dRP) termini. Further 5′dRP residue is excised by human DNA polymerase β associated with 5′dRP lyase activity, leaving 1 nucleotide gap with 5′. Further 1nt is synthetized by polβ followed by a final step, the nick sealing by ligases. Ligation during SN-BER occurs via DNA ligase III and scaffolding XRCC1 protein complex. This type of repair is named as a single nucleotide incorporation of BER repair (SN-BER). If the 5′dRP residue is oxidized, polβ becomes uneffective. Involving long-patch repair mechanism (LP-BER), 5′dRP is displaced with 2-8 additional nucleotides (nts) at the time of gap-filling synthesis as ssDNA flap, which is then removed by flap endonuclease 1 (FEN1). Further the ligation is performed by ligase I (Svilar et al. 2011, Hegde, Izumi, and Mitra 2012). LP-BER utilizes several enzymes from DNA replication pathways, such as pols δ or/and ε, the sliding clamp PCNA and clamp loader replication factor-C (RF-C) (Hegde, Hazra, and Mitra 2008). Recently it was discovered that ligase I rather than ligase III might be the major DNA ligase in both SN-BER and LP-BER. Ligase III mostly is essential for mitochondrial DNA (mtDNA) integrity (Gao et al. 2011, Simsek et al. 2011) Schematically BER pathways for repair of oxidized bases, AP sites and SSBs are represented in Figure 1.36.

PARP-1 is a key protein which has a role in DNA damage sensing and recruitment (De Vos, Schreiber, and Dantzer 2012, Gibson and Kraus 2012) In reaction of

oxidative damage, PARP1 binds to the lesions of DNA, such as breaks, nicks, gaps and stimulates the addition of ADP-ribose units as a post-translational modification . Moreover it was indicated that PARP1 is able to stimulate APE1 strand incision activity (Prasad et al. 2015). It is suggested that XRCC1 is dependent on PARP1 recruitment in order to remove the oxidative damage of purine base, such as 8-oxoG (Reynolds et al. 2015).

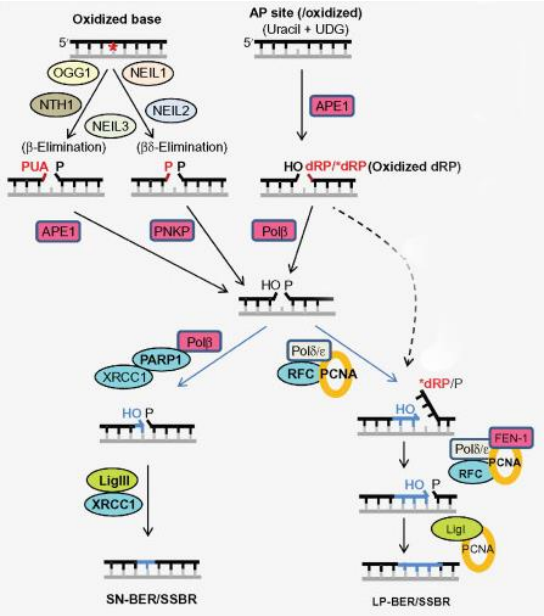


Figure 1.36 **Representation of BER pathways for repair of oxidized bases, AP sites and SSBs** (Hegde, Hazra, and Mitra 2008)

2. MATERIALS AND METHODS

2.1 Chemicals

Derivatives of 1,4-DHPs were synthesized in the Laboratory of Membrane Active Compounds at the Latvian Institute of Organic Synthesis. Structures of compounds are given in Supplementary material Table 1. Synthesis of the compounds was performed essentially as described (Dubur, Uldrikis, 1969). Tris base (TRIS), sucrose, ethidium bromide, acridine orange, Triton-X-100, Hind III/λ DNA digest, human serum albumin (HSA), ethidium bromide (EtBr), calf thymus DNA (ct-DNA) Na_2EDTA, LiCl, NaCl, $CaCl_2$ and other inorganic salts were purchased from Sigma-Aldrich (Taufkirchen, Germany). 2-mercaptoethanol was obtained from Ferak Berlin (Germany), sodium dodecyl sulphate was supplied by Acros Organics (Pittsburg, USA), isoamylic alcohol was obtained from Stanlab (Lublin, Poland), and 6×Orange loading solution, RNase A and Proteinase K were purchased from Thermo Fisher Scientific (Pittsburg, USA).

2.2 Cell culture

HeLa cells (Biomedical Research and Study Centre, Riga, Latvia) were grown in DMEM + GlutamaxTM – I, F-12 Nut-Mix (1x) (Sigma-Aldrich, Taufkirchen, Germany) + 10% fetal bovine serum (Sigma-Aldrich, USA), at 37°C in a humidified atmosphere containing 5% CO_2. In some experiments, the cells were transfected with plasmid (pCI-NeoDUX4) encoding DUX4 protein and GFP protein as a transfection control, using jetPEI Polyplus transfection solution (Illkirch, France).

2.3 UV/VIS spectroscopic measurements

UV-VIS spectra of compounds were recorded with a Perkin Elmer Lambda 25 UV/VIS spectrophotometer in the absence and in the presence of increasing amounts of DNA bases (A,T,G,C) in 5 mM Tris HCl and 50 mM NaCl buffer at pH 7.4 or other. A 25 μM solution of the tested compound was diluted out of a 1mM stock solution in the buffer in a quartz cell (2 ml). A reference cell was filled with 2 ml of the buffer. The mixture was mixed thoroughly and titrated by base solution, 10 μM each time to both sample and reference cells. Spectra were recorded in a 400-200 nm interval at room temperature.

Binding constants (K_b) were calculated by applying the formula:

$$\frac{1}{A_0 - A} = \frac{1}{A_0} + \frac{1}{K \times A_0 \times c_{\mathrm{DNA}}}$$

according to (Zhang et al. 2011), where A_0 is absorption of the free substance, A is absorption in presence of DNA base, and c_{DNA} is base concentration.

2.4 Fluorescence spectroscopic measurements

Spectrofluorometric analyses were performed on a Fluoromax-3 (Horiba JOBIN YVON, China). ct-DNA was previously sonicated for induction of random DNA breaks. Fluorescence spectra of a 25 μM solution of the 1,4-DHP in 50 mM Tris-HCl, 50 mM NaCl at pH 7.4 or other buffer were recorded over a range of 365-600 nm depending on excitation wavelength of compound. DNA was sequentially added 12.5 μM at each step. K_b were evaluated from intercept by plotting log $[(F-F_0)/F$ vs. log[DNA] using following equation:

$$\log(F-F_0)/F = \log Kb + n\log[DNA],$$

where, F_0 and F are fluorescence emission intensity before and after DNA addition, K_b is the binding constant and the binding site size (n), which was evaluated from the slope of the plot (Arshad et al. 2017). In other experiments, titration was performed by human serum albumin solution (HSA), 5μM each time. Fluorescence spectroscopic experiments on the interaction of 1,4-DHP with the DNA-EtBr complex were carried out at room temperature in 5 mM Tris HCl; 50 mM NaCl at pH 7.4 or other buffer using a 1-cm cuvette. The complex of ct-DNA (74.8 μM) and EtBr (1.26 μM) was titrated with 8.3 μM aliquots of the 2.5 mM solution of the compound. After each titration, the solution was thoroughly mixed and allowed to equilibrate for 5 min prior to fluorescence measurement. Fluorescence intensity of the EtBr-DNA complex was recorded at 600 nm using an indirect excitation wavelength at 260 nm (Geall and Blagbrough 2000). Quenching constants were described by the linear Stern-Volmer equation in which derivatives of 1,4-DHPs were the quenchers:

$$I_0/I = 1 + K_{sv}[Q],$$

where I_0 and I represent the fluorescence intensities in the absence and presence of quencher, respectively; K_{sv} is a linear Stern-Volmer constant, Q is the concentration

of quencher. K_{sv} values were evaluated from the slope of the plot (Geethanjali et al. 2015).

2.5 Circular dichroism spectroscopy

Circular dichroism (CD) spectra were recorded on a Chirascan CS/3D spectrometer (Applied Photophysics, Surrey, UK); ct-DNA and compound binding measurements were done in 10 mM HEPES (Sigma-Aldrich, Germany) buffer, pH 7,4 in a quartz cell of 10 mm path-length at room temperature. CD spectra of ct-DNA were recorded in a range of 200-300nm, spectra of compound in a range of 300-420 nm and spectra of human serum albumin in a range of 200-260 nm. The parameters for all spectra were as follows: scan rate - 200 nm min^{-1}, averaging time - 0.125 s, bandwidth - 1 nm; one recorded spectrum is the average of four scans. CD spectra of HSA in the absence or in the presence of AV-153 salts were recorded in PBS buffer, pH 7,4. A 300 nM HSA solution was titrated with AV-153-Na (1 μM at each step). Titration in the ct-DNA region was carried out by adding progressively increasing amounts of AV-153 salts (10 μM at each step) to 50 μM sonicated ct-DNA solution. Titration in the induced CD region of the compound was performed by adding DNA (62.5 μM at each step) to 500 μM AV-153-Na solution.

2.6 The single cell gel electrophoresis

Cells in the exponential phase of growth were washed with 1x Dulbecco`s phosphate buffer (PBS) without glucose, $MgCl_2$, $CaCl_2$ (Sigma-Aldrich, Germany). The medium was replaced by phosphate buffer (50 mM Na_2HPO_4, 90 mM NaCl, 5 mM KCl, 0.1 mM $CaCl_2$, 8 mM $MgCl_2$, 5 mM glucose, pH 7.4) and 1,4-DHPs were added to the buffer (0 - 100 nM). Incubations lasted for 45 min at 37 ^oC in a humidified atmosphere containing 5% CO_2. After incubation cells were washed in PBS and the bolus of peroxynitrite (6 μl) was added at a final concentration of 200 μM. During the peroxynitrite treatment, the cell plate was gently swirled for 15 s. The action was repeated 2 times (total duration of exposure to peroxynitrite 30 s). To assess the cell protection against peroxynitrite in the presence of the studied DHPs, the compounds were added before the peroxynitrite treatment or simultaneously with it. After the incubations, cells were washed in PBS, trypsinized and put on comet assay. To assess the impact of alkaline medium on DNA breaks, group of vehicle control was introduced (bolus of 10 mM NaOH, 6 μl, final concentration 60 μM). The comet

assay was performed as described (Tice et al. 2000, Ryabokon et al. 2005) with minor modifications (L Olive and Banáth 2006). HeLa cells treated or not treated with peroxynitrite in absence or presence of DHP and were detached by trypsinization. 50 µl of cell suspension containing 10 000 cells were mixed with 100 µl of 1% low melting-point agarose (Sigma-Aldrich, USA) and placed on a microscope slide that had been pre-coated with 0.5% normal melting-point agarose. The cell membranes were lysed by keeping the slides in cold lysing solution (pH 10.0) that contained 2.5 M NaCl, 10 mM Na_2EDTA, 10 mM Tris (AppliChem, Germany), 1% Triton-X 100, 5% DMSO (Sigma-Aldrich,USA), for at least 1 h. Subsequently, the slides were placed in a horizontal tank filled with fresh electrophoresis buffer (1 mM Na_2EDTA, 300 mM NaOH, pH 13.2) for 20 min to allow DNA to unwind. Then, horizontal electrophoresis was carried out for 20 min at 300 mA, 1 V/cm and 4° C. After electrophoresis, slides were washed twice for 5 min with 0.4 M Tris buffer (pH 7.5) for neutralization and then fixed in ice-cold 96 % ethanol for 10 min. Slides were dried and stained with ethidium bromide and analyzed with a fluorescence microscope equipped with 515–560 nm excitation filter and 590 nm barrier filter.

Cells were visually graded into 5 classes ($A_0 – A_4$) (Ryabokon et al. 2005) from class 0 (undamaged, no discernible tail) to class 4 (almost all DNA in tail, insignificant head). The mean value of DNA damage (D) in arbitrary units was calculated as follows: $D = A_1 + 2 \times A_2 + 3 \times A_3 + 4 \times A_4$

2.7 UV/VIS spectroscopic measurement of peroxynitrite decomposition.

The rate of peroxynitrite (0.38 mM) decomposition in the presence or in the absence of the 1,4-DHP (0.16 mM) was followed at 302 nm (absorbance peak for the peroxynitrite anionic form) in 10 mM Tris pH 10 buffer on Perkin Elmer Lambda 25 UV/VIS spectrophotometer (Carballal, Bartesaghi, and Radi 2014). The average rates of reactions were calculated according to the formula $V = \pm ((C_2 – C_1) / (t_2 - t_1)) = \pm (\Delta C/ \Delta t)$, where C_1 was the concentration of peroxynitrite in the beginning of reaction, and C_2 the concentration of peroxynitrite at the end of the reaction; Δt: 20 min.

2.8 Statistical analysis

The values of DNA damage assayed by single gel electrophoresis are represented as the mean ± standard error of the mean (SEM). The data were subjected to the one-way

analysis of variance (ANOVA), followed by the Tukey multiple comparisons test and the data were considered as significant at $p < 0.05$.

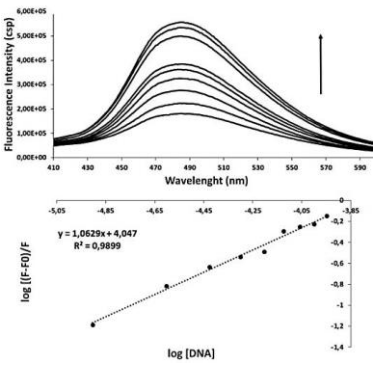
3. RESULTS

3.1 Fluorescence spectroscopic measurements: Fluorescence titration

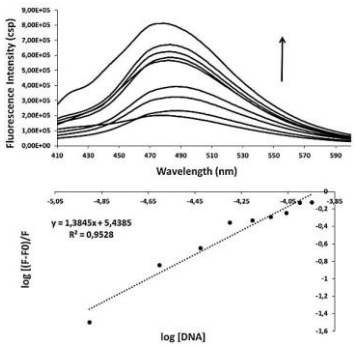
3.1.1 Interaction of AV-153 salts with ct-DNA

Fluorescence spectra of AV-153 salts with different metal ion in position 4 are shown in Figure 3.1. Exitation and emission wavelengths of compounds are presented in Table 1. Fluorescence intensity (FI) of AV-153 salts noticeably increased after each addition of sonicated ct-DNA to the solution of the compound. Red shift (batochromic effect) for Li salt and blue shift (hypsochromic) for Rb salt was also observed, indicating structural changes of DNA. As indicated by the calculation of binding constant (K_b) and binding site size (n) the intensity of binding was dependent on the metal ion forming in position 4 (Table 1). The affinity of the AV-153 derivatives decreased in the following order Mg>Na>Ca>Li>Rb>K. n which reflects binding of the compound per base between AV-153 salts was also varying. n of the Na, Ca and Mg salts were evaluated as $n \geq 1$, and the values of Li, K and Rb were evaluated as $n \leq 1$. According Arshad et. al, if the n of the compound and DNA complex equals one, this indicating the intercalative mode of binding; if the site size is significantly higher than one, this points to the mixed binding mode including intercalation, groove binding and electrostatic interactions; if the binding site is smaller than one, this points to an exclusively external binding via groove or electrostatic binding (Arshad et al. 2012, Arshad et al. 2017). Thus, it turns out that Mg, Ca and Na salts interact with DNA via intercalation, and probably groove binding; Li, Rb and K salts bind to DNA externally.

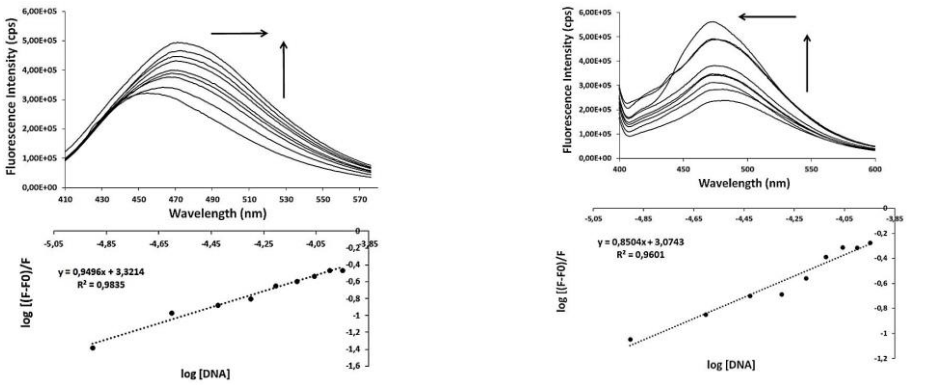
AV-153-Ca



AV-153-Mg



AV-153-Li **AV-153-Rb**



AV-153-Na **AV-153-K**

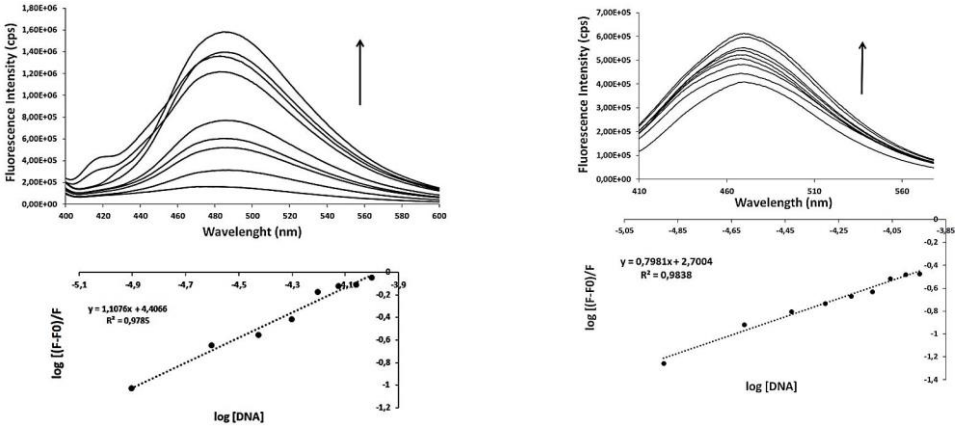


Figure 3.1 **Fluorescence titration of AV-153 salts with sonicated ct-DNA**. Spectra of intrinsic fluorescence of AV-153 salts were recorded before or after DNA addition, concentration of DNA was increased for 12.5 μM each step. Arrows indicate direction of the fluorescence intensity and maxima position changes with addition of DNA solution to the solution of the compound. Plots log [(F-F_0)/F vs. log[DNA] used for calculation of *Kb* and *n* are given below the spectra.

Table 1. **Affinity of AV-153 salts to ct-DNA determined by spectrofluorimetry**

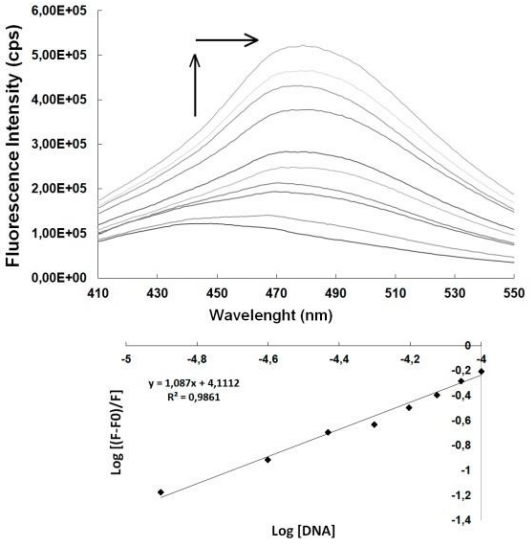
Compound	**R_3=R_5**	**R_4**	**(ex./em.)**	**Fluorescence intensity**	**Spectrofluorimetrically determined binding constant, M^{-1}**	**Binding site size (n)**
AV-153-Na	-$COOCH_2CH_3$	-COONa	350/480	↑	4.40 x 10^5	1.10

AV-153-Mg	-$COOCH_2CH_3$	$(COO^-)_2$	350/480	↑	5.43×10^5	1.38
AV-153-Ca	-$COOCH_2CH_3$	$(COO^-)_2$	350/480	↑	4.04×10^5	1.06
AV-153-Rb	-$COOCH_2CH_3$	-COORb	350/470	↑/ blue shift (8 nm)	3.07×10^5	0.85
AV-153-Li	-$COOCH_2CH_3$	-COOLi	350/455	↑/ red shift (21 nm)	3.32×10^5	0.94
AV-153-K	-$COOCH_2CH_3$	-COOK	350/470	↑	2.70×10^5	0.80

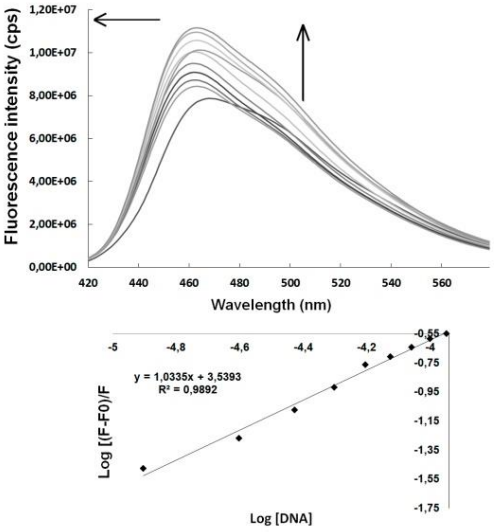
3.1.2 Interaction of other 1,4-DHP derivatives with ct-DNA

Affinity to DNA of another synthetic derivatives of 1,4-DHPs using fluorescence titration assay was also detected. FI changes of the compounds in the presence of DNA are shown in Figure 3.2. Binding constants and binding site size are presented in Table 2. Among tested 1,4-DHPs, changes of FI of compound (increase, decrease or shift) after DNA edition were detected for J-4-96, FI considerably increased after DNA addition with a large red shift (~40 nm) of emission maximum. PP-544-NH4, which is a representative of tricyclic derivatives of 1,4-DHP-decahydroacridine-1,8-diones, have a high affinity to DNA. The emission maxima of other representatives of this group PP-150-Na and B-5- shifted to a red side, but without the fluorescence intensity changes, however this effect could point to DNA-compound complex formation and changes in DNA conformation. Tauropyrone showed itself as an active DNA binder. Among carbatonides (etcarbatone, metcarbatone, carbatone, propcarbatone and styrylcarbatone) the only styrylcarbatone (J-9-125) manifested FI decrease in the presence of DNA. Among lipophilic derivatives of 1,4-DHPs (cerebrocrast, etaftorone and fenoftorone) only the fluorescence of cerebrocrast changed significantly, decreasing by the addition of DNA.

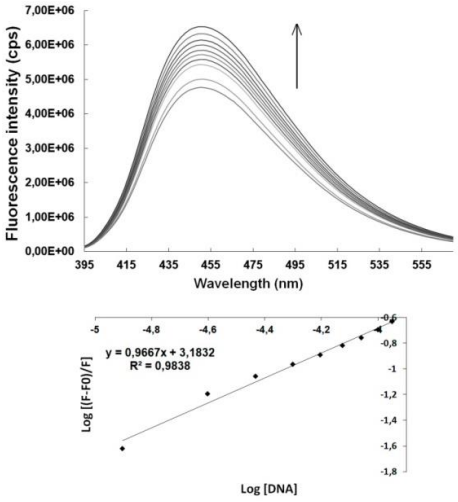
J-4-96



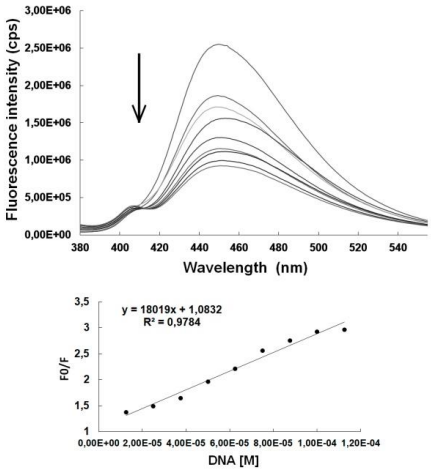
PP-544-NH_4



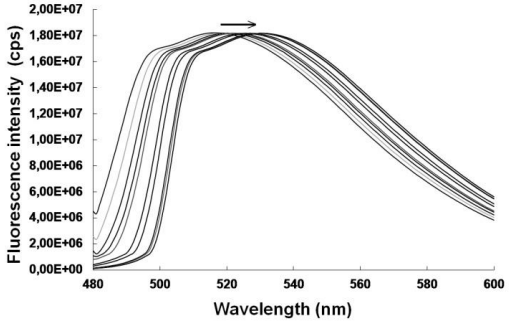
Tauropyrone



Styrylcarbatone (J-9-125)



PP-150-Na



Cerebrocrast

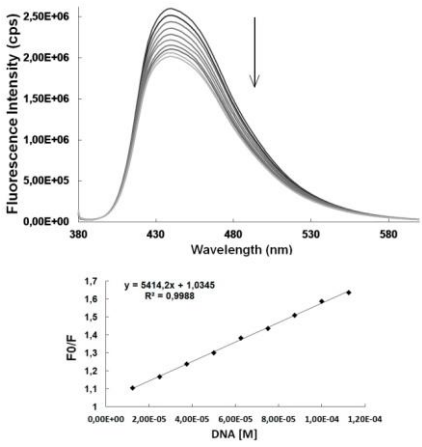
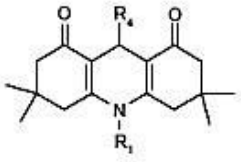
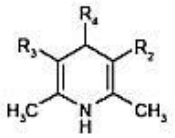


Figure 3.2 **Fluorescence titration of 1,4-DHPs with ct-DNA**. Spectra of intrinsic fluorescence of 1,4 –DHP derivatives were recorded before or after DNA addition. DNA was added by 12.5 μM at each step. Arrows indicate direction of the fluorescence intensity and maxima position changes with addition of the DNA solution. In case of fluorescence increase plots log [(F-F_0)/F vs. log[DNA] was used for calculation of K_b and *n*. In case of fluorescence decrease Stern Volmer plot was used for calculation of binding constant (*Ksv*) and *n.*

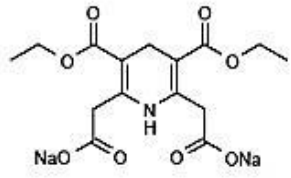
Table 2. **Affinity of 1,4-DHP derivatives to ct-DNA determined by spectrofluorimetry**



1-4



5-16



17

1.PP-544-NH_4: R_1 =H, R_4=COONH4
2. PP-544-Na: R_1=H, R_4 = COONa
3. PP-150-Na: $(CH_2)_5$COONa, R_4=Ph
4. B-5-Na: R_1 = CH_2CH_2COONa, R_4=Ph

Nr.	Compound	R_2=R_3	R_4	Ex/Em (nm)	Changes in fluorescence intensity	Spectrofluorimetrically determined binding constant, M^{-1}	Binding site size (n)
1	PP-544-NH_4 (J-6-94)	See insertion	See insertion	380/470	↑/blue shift	3.5 x 10^5	1.03
2	PP-544-Na	See insertion	See insertion	380/470	NC		
3	PP-150-Na	See insertion	See insertion		NC/red shift 15 nm		
4	B-5-Na	See insertion	See insertion		NC/red shift 10 nm		
5	Cerebrocrast	-$COOC_2H_4OC_2H_7$	$C_6H_5OCHF_2$	350/440	↓	5.4 x 10^3 (K_{sv})	1.03
5	Etaftorone	-$COOC_2H_4OC_2H_5$	$C_6H_5OCHF_2$	350/440	NC		
7	Fenoftorone	-$COOC_2H_4OC_6H_5$	$C_6H_5OCHF_2$		NC		
8	Etcarbatone	-$COHOCH_2COONa$	C_2H_5	360/450	NC		
9	Metcarbatone	-$COOCH_2COONa$	CH_3	360/450	NC		

10	Carbatone	$-COOCH_2CH_3$	-H	380/460	NC		
11	Propcarbatone	$-COOCH_2COONa$	C_3H_4-n	360/450	NC		
12	Styrylcarbatone (J-9-125)	$-COOCH_2COONa$	$C_2H_4C_6H_5$	350/450	↓	1.8×10^4 (K_{sv})	1.08
13	Tauropyrone	$-COOCH_2CH_3$	$-CONHCH_2CH_2SO_3Na$	350/450	↑	3.1×10^5	0.97
14	Alapyrone	$-COOCH_2CH_3$	$-CONHCH_2CH_2COOH$		NC		
15	AV-154-Na	$COCH_3$	-COONa	350/470	NC		
16	J-4-96	$-COOC_2H_4CH_3$	-COONa	350/440	↑/red shift	4.1×10^5	1.09
17	J-131-Na	See insertion	See insertion	350/470	NC		

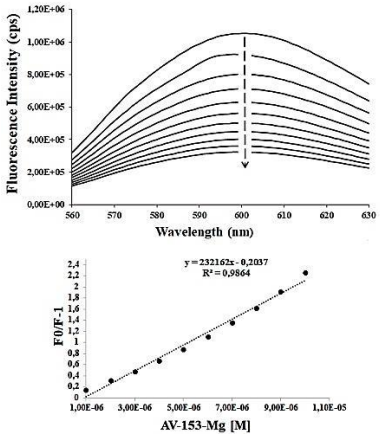
* NC – no change

3.2 Fluorescent intercalator displacement assay

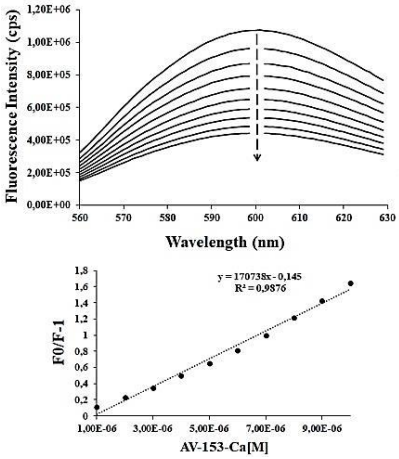
3.2.1 Interaction of AV-153 salts with EtBr-DNA complex

The enhanced fluorescence of the DNA-EtBr complex was quenched by the addition of AV-153 salts. As presented in Figure 3.3, all salts of AV-153 considerably decreased the fluorescence of DNA-EtBr complex. Calculation of Stern-Volmer quenching constants (K_{sv}) revealed that AV-153-Mg and AV-153-Ca were more competitive than Na, Li and K salts. Rb salt salt has the weakest intensity. The competitive effects of compounds and EtBr decreased in following order Mg>Ca>Li>Na>K>Rb.

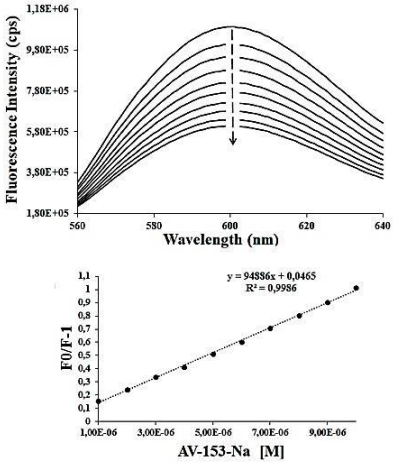
AV-153-Mg



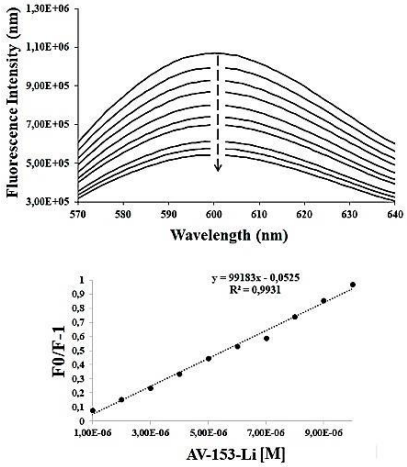
AV-153-Ca



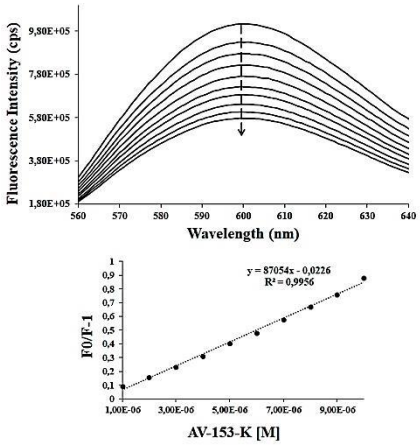
AV-153-Na



AV-153-Li



AV-153-K



AV-153-Rb

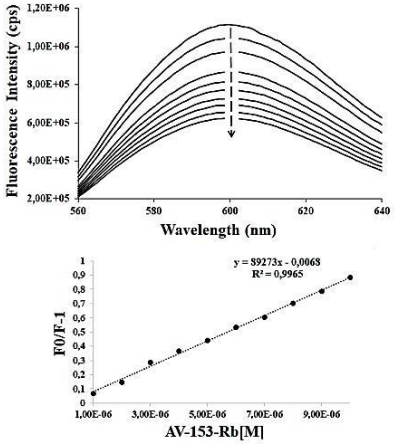


Figure 3.3 **Fluorescence spectra of EtBr-DNA complex in the presence of increasing concentration of AV-153 salts.** Concentration of EtBr was 1.26 μM, DNA-74,8 μM. AV-153 salts were added by 10 μM at each step 10 times. Stern Volmer plots F0/F-1 vs. concentration of quencher (AV-153 salts) was used for calculation of quenching constant (K_{SV}) are given below the spectra.

Table 3. **The ability of the AV-153 salts to extrude the EtBr from intercalation sites**

Compound	Ksv, M^{-1}	Fluorescence decrease, % (+100μM of compound)	Correlation coefficient
AV-153-Na	9.4×10^4	44	.99
AV-153-Ca	1.7×10^5	60	.99
AV-153-Mg	2.3×10^5	65	.99
AV-153-Li	9.9×10^4	50	.99
AV-153-K	8.7×10^4	42	.99
AV-153-Rb	8.9×10^4	45	.99

3.2.2 Interaction of another 1,4-DHPs with EtBr-DNA complex

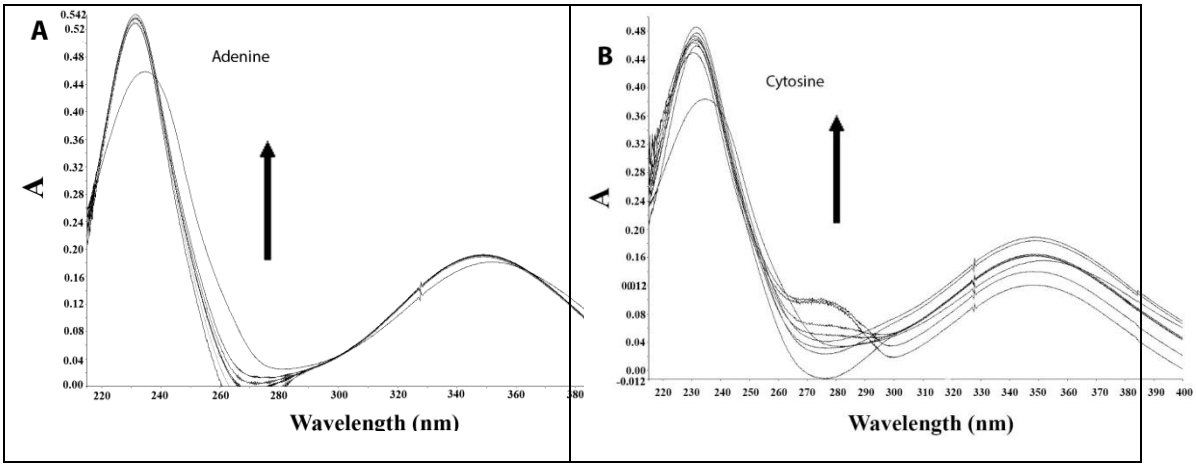
Another derivatives of 1,4 –DHPs, which demonstrated the ability to interact with DNA (PP-544-NH_4, cerebrocrast, styrylcarbatone, J-4-96 and tauropyrone) were also tested for ability compete with EtBr. Stern Volmer quenching constants (K_{sv}) and changes of fluorescence intensity of EtBr-DNA complex after addition of compounds are presented in Table 4. The enhanced fluorescence of the DNA-EtBr complex was remarkably quenched by the addition of styrylcarbatone (J-9-125), whose K_{sv} was close to intercalator constants. PP-544-NH_4 also manifested the ability to replace the EtBr and to quench the fluorescence of EtBr-DNA complex. J-4-96 and cerebrocrast produced very weak impact on displacement of EtBr and reduced the fluorescence intensity of ETBr-DNA complex only for 5 and 12% ,correspondingly. Tauropyrone did not affect the fluorescence intensity of EtBr-DNA complex. It seems that the major binding mode of these three compounds is a groove binding.

Table 4. **The ability derivatives of 1,4-DHP to extrude the EtBr from intercalation sites**

Compound	Ksv, M^{-1}	Fluorescence intensity decrease of EtBr-DNA complex, % (+100μM of compound)	Correlation coefficient
PP-544-NH_4	8.6 x 10^4	40%	0.99
Cerebrocrast	3.9 x 10^3	5%	0.98
Styrylcarbatone (J-9-125)	1.7 x 10^5	70%	0.99
J-4-96	1.7x 10^4	12%	0.98
Tauropyrone		0%	

3.3 Interaction of AV-153-Na with bases of DNA

The influences of DNA bases, C, G, A and T on the UV/VIS absorption spectra of AV-153-Na were used to evaluate possible base-specificity of binding. Data are presented in Figure 3.4 and Table 5. The absorption intensity was gradually increased with the increase of the concentration of the four bases. Affinity to G, C and T is greater than that to A. The results indicate that AV-153-Na may interact with the four types of bases, with somewhat different affinities. In order to evaluate the role of ionic and hydrogen bonds in AV-153-Na interactions with bases, titration was performed in solutions of 1M NaCl and 8M urea. In these, media affinity of AV-153-Na to bases was weakened, especially for G; the shape of spectra was also changed. However, interactions were not abolished.



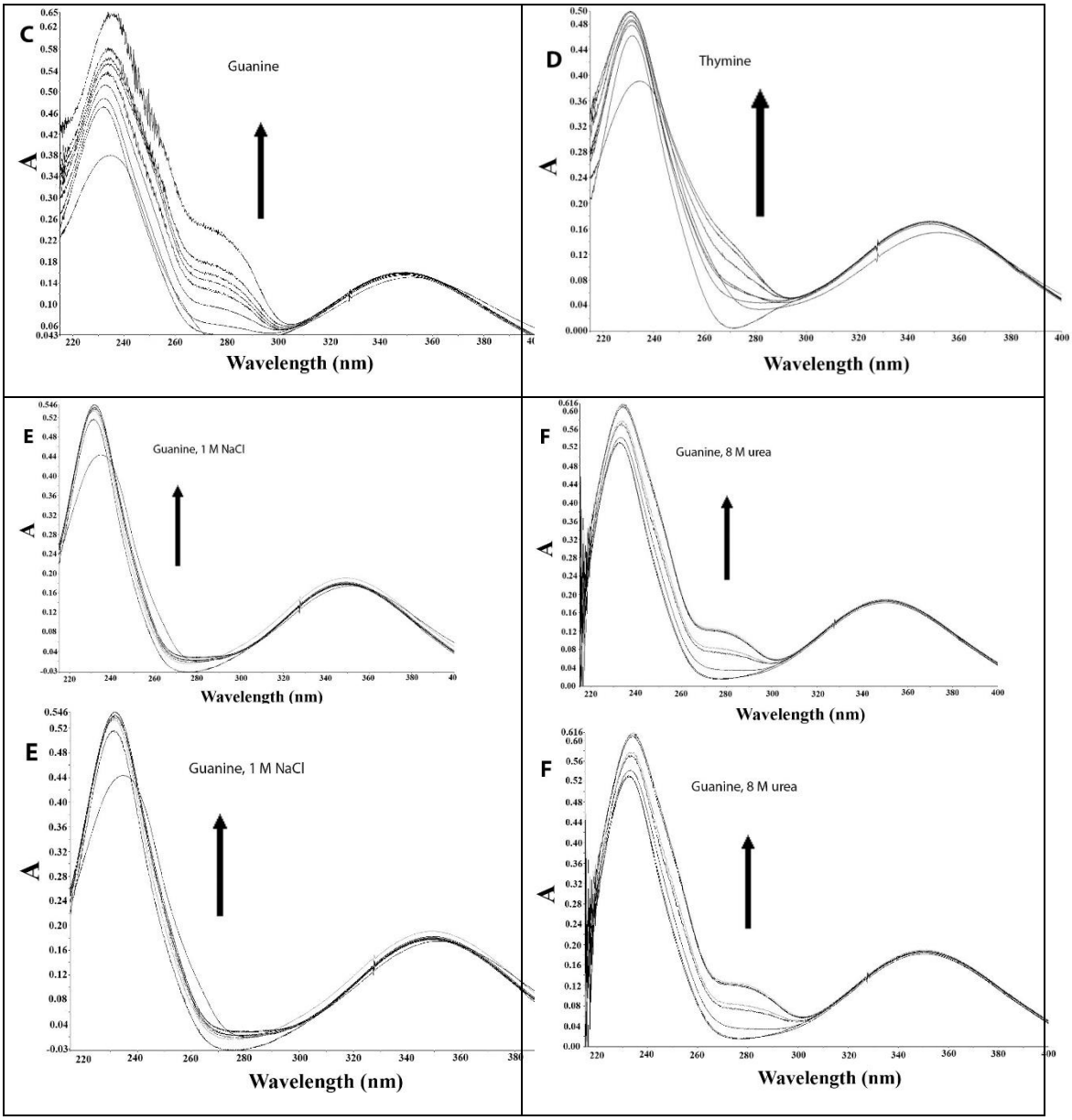


Figure 3.4 **AV-153-Na absorption spectra in absence and presence of bases in different solutions.** Concentrations of bases were increased for 10 μM with each titration. A – Adenine, B – Cytosine, C, E, F – Guanine, D – Thymine. A – D – in 5 mM Tris HCl, pH 7.4, 50 mM NaCl; E – 1 M NaCl; F – 8 M urea.

*Table 5.***Affinity to bases of AV-153-Na**

Base	Binding constants of the AV-153 salts in different media		
	AV-153-Na, 5 mM Tris-HCl, 50 mM NaCl	**AV-153-Na, 1 M NaCl**	**AV-153-Na, 8 M urea**
Adenine	2.6×10^3	2.3×10^3	2.5×10^3
Cytosine	3.6×10^3	2.8×10^3	3.0×10^3

Guanine	8.8×10^3	3.2×10^3	1.9×10^3
Thymine	3.5×10^3	5.11×10^3	3.1×10^3

3.4 Circular dichroism analysis of AV-153-Na and ct-DNA binding

Circular dichroism spectra of ct- DNA in presence of increasing concentrations of AV-153-Na are shown on Fig 3.5A. DNA manifests a negative band at 245 nm due to helicity and positive band at 270 nm because of base stacking which is characteristic of the B form of DNA. Adding AV-153-Na to DNA increases the negative band intensity and decreases the positive band intensity. A 2 nm red shift of crossover point is also observed. These data clearly indicate interactions of the compound with DNA, although changes in spectra are not typical for any binding mode. Induced circular dichroism experiment, when measurements were done in the 1,4-DHP absorbance area with fixed concentration of the drug and increasing concentrations of DNA, a negative band with maximum at 340 nm was observed, its intensity increased with each portion of added DNA, a red shift of maximum was also observed. (Fig. 3.5B). The increase of negative ICD signal in the region of compound absorbance spectra after DNA addition usually points to intercalative-binding mode (Garbett, Ragazzon, and Chaires 2007).

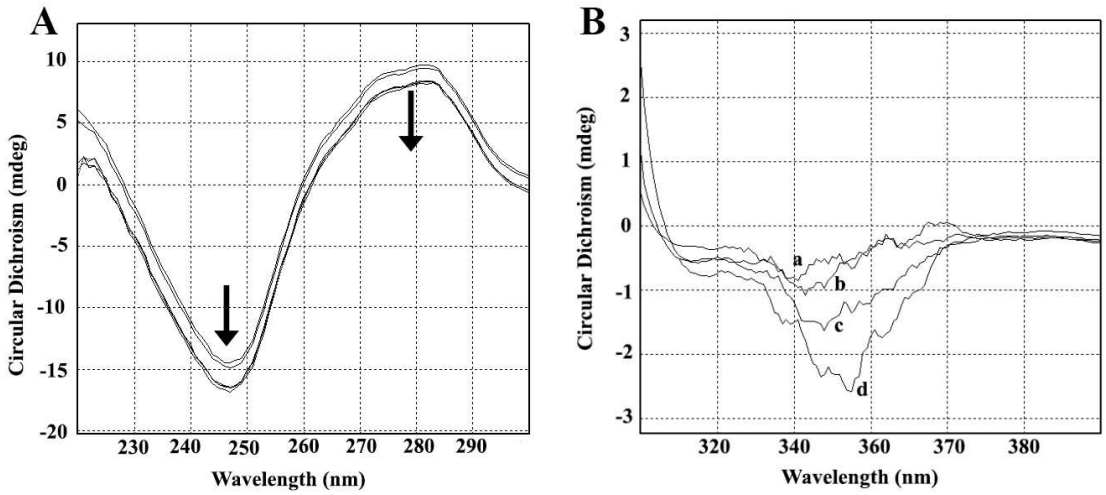


Figure 3.5. **Circular dichroism experiments on interaction of AV-153-Na and ct-DNA.** A - Circular dichroism spectra of ct- DNA in absence and presence of AV-153-Na. AV-153-Na concentration was increased by 10μM at each step up to 40μM. DNA concentration was 50 μM. Measurements were performed in 10 mM HEPES buffer. B – Induced circular dichroism

spectra of AV-153-Na CD spectra (500 μM) in presence of 62.5 μM(a), 125 μM (b); 250 μM (c) and 500 μM of DNA. Measurements were performed in HEPES buffer.

3.5 Interactiom of AV-153 salts with human serum albumine

Fluorescence spectrum of AV-153-Na alone and in the presence of human serum albumin (HSA) is presented in Figure 3.6. Fluorescence of AV-153-Na significantly increased in the presence of HSA with a large blue shift. Binding constant (K_b) was equal to 3.9 x 10^5, that points to a high affinity of AV-153-Na with HSA. The binding sites (n) per 1 BSA molecule was 0,93 (n≤1) , that means what the AV-153-Na has the only one independent binding site per one HSA molecule. Results obtained by circular dichroism revealed that AV-153-Mg, Ca and Rb salt have an affinity to DNA. BSA manifests two negative bands at UV region (208 and 222 nm) that is peculiar to α-helical structure of protein. ICD signal decreased in both bands after each addition of compound indicating about α-helical content decrease due to conformational changes of HSA molecule (Fig 3.7) (Varlan and Hillebrand 2010).

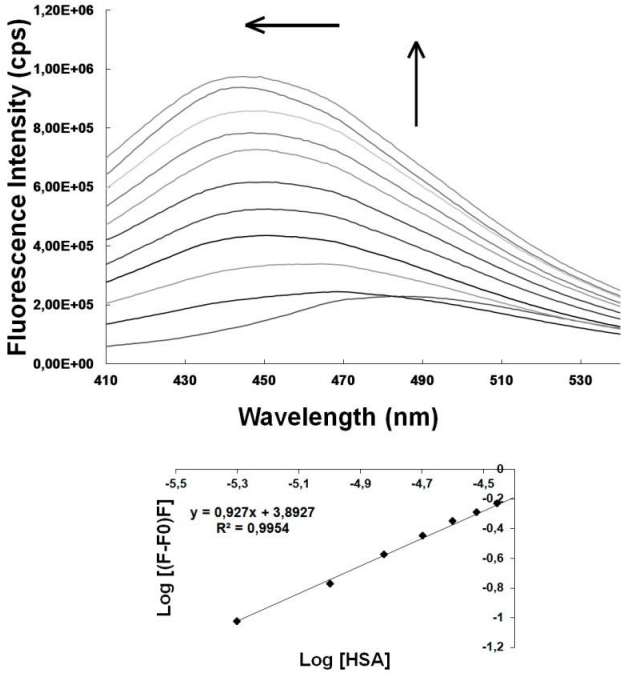


Figure 3.6 **Fluorescence titration of AV-153-Na with human serum albumin.** 5 μM of HSA was added each time. Arrows indicate direction of the fluorescence intensity and maxima position changes with addition of the HSA to solution of AV-153-Na. Plot log [(F-F_0)/F vs. log[HSA] was used for calculation of K_b and *n* are given below the spectra.

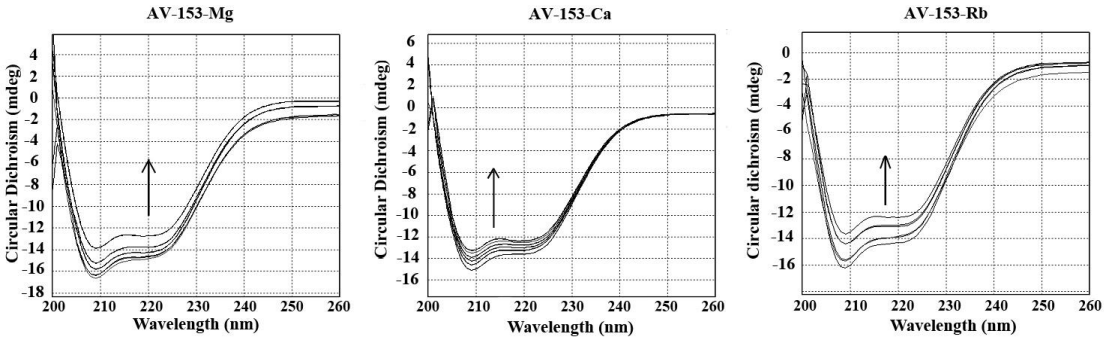


Figure 3.7 **CD spectra of HSA in the absence or in the presence of AV-153 salts**.
300 nM HSA solution titrated with AV-153 salt. AV-153 salts concentration was increased by 1μM at each step. Measurements were performed in PBS buffer, pH 7.

3.6 The decomposition rate of peroxynitrite in the presence of derivatives of 1,4-DHP

3.6.1 AV-153 salts

The ability of the AV-153 salts to degrade peroxynitrite chemically was tested by kinetics of decomposition of peroxynitrite in the presence of DHP followed by spectrophotometry (Figure 3.8). The average rate of decomposition of peroxynitrite at a concentration of 0.38 mM was 0.0157 μmol/μl·min. The decomposition rate of peroxynitrite increased in the presence of AV-153-Mg: 0.0391 μmol/μl·min, AV-153-Li: 0.0181 μmol/μl·min and AV-153-Ca: 0.0179 μmol/μl·min, that may indicate that these compounds can react with peroxynitrite and cause its decomposition. Other AV-153 salts did not affect time of decomposition of peroxynitrite, AV-153-K: 0.0163 μmol/μl·min, AV-153-Rb: 0.0156 μmol/μl·min.

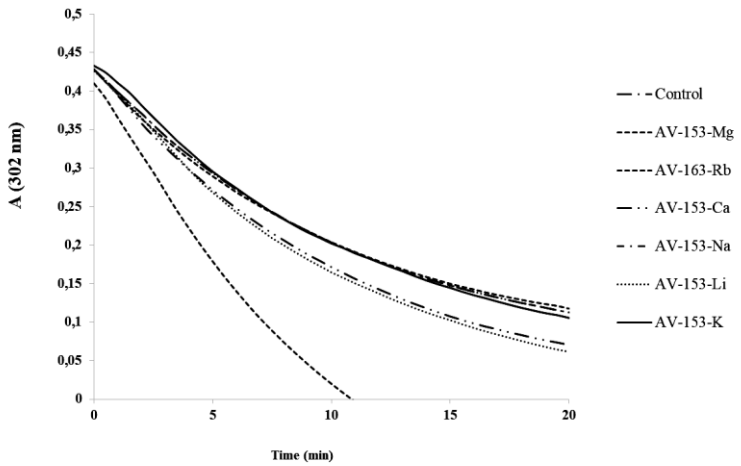


Figure 3.8 **Decomposion of peroxynitrite in the presence of the AV-153 salts.** Peroxynitrite was added to the sample cuvette (0.38 mM in TrisHCl, pH 10) and its

decomposition with time was followed at 302 nm. To test the impact of the AV-153 salts on the process, the compounds were added to both sample and control cuvettes up to 0.16 mM before adding the peroxynitrite.

3.6.2 Another derivatives of 1,4-DHP

Another 9 derivatives of 1,4-DHPs were tested for ability to enhance the decomposition rate of peroxynitrite. The decomposition rates of peroxynitrite in the presence of the compounds are presented in Table 6. All water soluble 1,4-DHPs did not accelerate the decomposition rate of peroxynitrite. Meantime all lipophilic derivatives (cerebrocrast, etaftorone, fenoftorone) of 1,4-DHP enhanced the breakdown of peroxynitrite (Fig 3.9). Cerebrocrast was the most effective; it accelerated the rate of peroxynitrite decomposition for 8 times. Etaftorone and fenoftorone speed up the rate for 2.5 and 2.3 times.

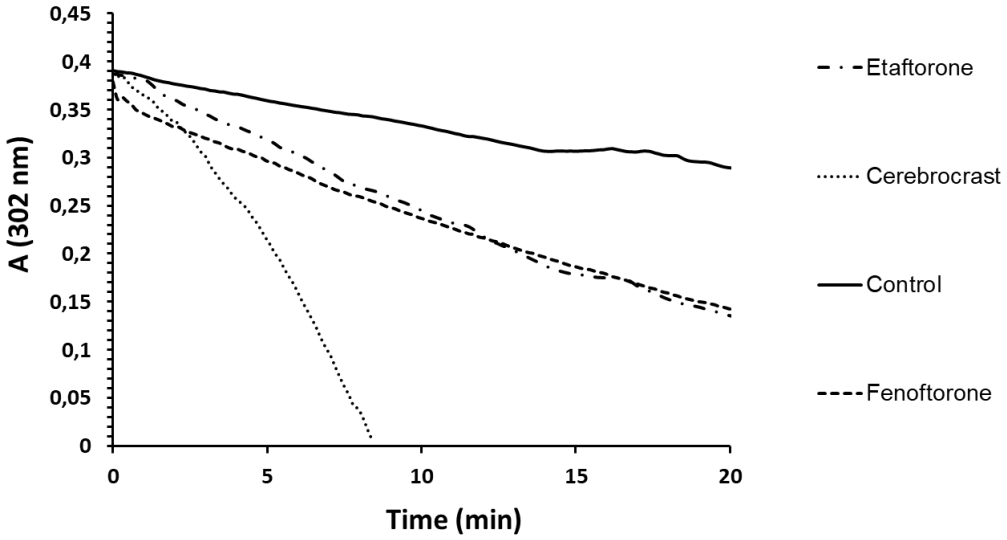


Figure 3.9 **Decomposion of peroxynitrite in the presence of the etaftorone, cerebrocrast and fenoftorone.** Peroxynitrite was added to the sample cuvette (0.38 mM in TrisHCl, pH 10) and its decomposition with time was followed at 302 nm. To test the impact of the compounds on the process, the compounds were added to both sample and control cuvettes up to 0.16 mM before adding the peroxynitrite.

The strong DNA binder PP-544-NH_4 produced a paradoxical kinetic effect- the optical density of peroxynitrite in the presence of this compound increased with time. Apparently PP-544-NH_4 interact chemically with peroxynitrite and reaction product has absorbance peak in the area of peroxynitrite absorbance maximum (Fig 3.10)

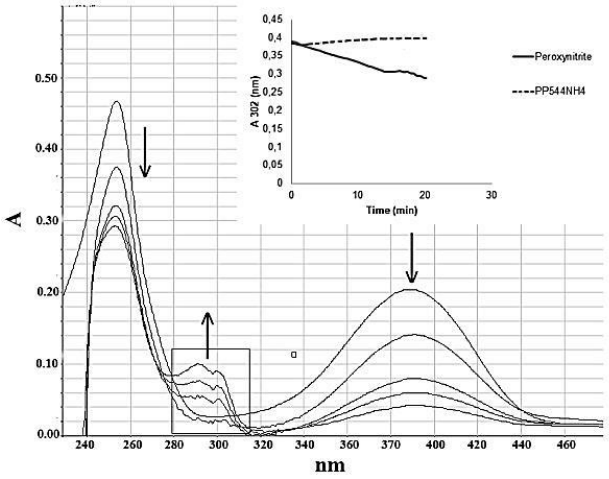


Figure 3.10 **Changes of the spectrum of PP-544-NH_4 in the peresence of peroxynitrite with time.** Spectrum was recorded of PP-544-NH_4 every 5 minutes. Peroxynitrite was added also to the control cuvette.

Table 6. **The average rate of peroxynitrite decomposition in the presence of 1,4-DHP derivatives**

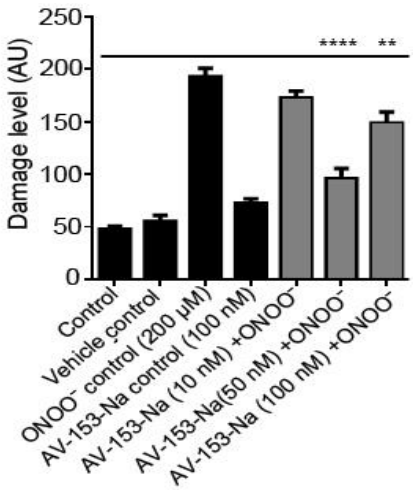
Compound	The decomposition rate of peroxynitrite (μM/μl · min)
Peroxynitrite (control)	0,0056
Etaftorone	**0.0138**
Cerebrocrast	**0.0453**
J-8-120	0.0027
J-3-186	0.0055
J-7-53-B	0.0014
Fenoftorone	**0.0129**
AV-154-Na	0.0033
Etcarbatone	0.0038
Metcarbatone	0.0013

3.7 The ability to protect DNA against peroxynitrite in living cells

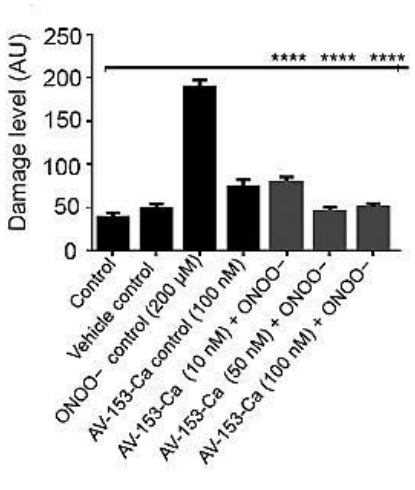
3.7.1 AV-153-salts

DNA-protecting action of AV-153 salts against peroxynitrite-induced damage was tested in living HeLa cells. Cells were treated with peroxynitrite alone or after incubation with AV-153 salts and then the level of DNA damage was analysed using the Comet assay (Fig. 3.11). Treatment with peroxynitrite drastically increased the levels of DNA damage. AV-153-Ca turned out to be the most effective, it decreased the level of DNA damage almost four-fold, when the cells were pre-incubated with 10, 50 and 100 nM of the compound for 45 min. Higher concentrations (50 and 100 nM) were effective even when the compound was given simultaneously with peroxynitrite, other compounds did not protect DNA when given simultaneously with the peroxynitrite (Supplementary material Fig. 1). AV-153-Rb produced much weaker effects when pre-incubation was performed with lower concentrations (10 and 50 nM). AV-153-K produced some protecting effect when applied in 10 nM concentration. AV-153-Li was not effective at all, but AV-153-Mg induced some DNA damage itself.

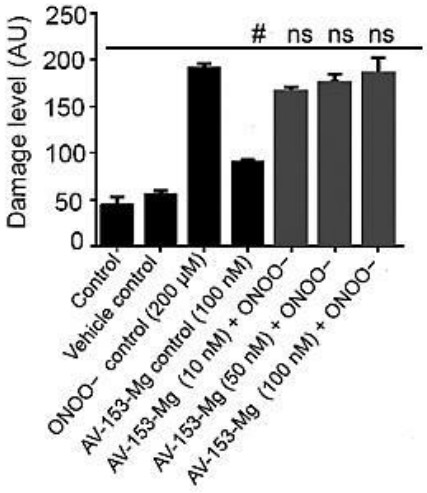
AV-153-Na



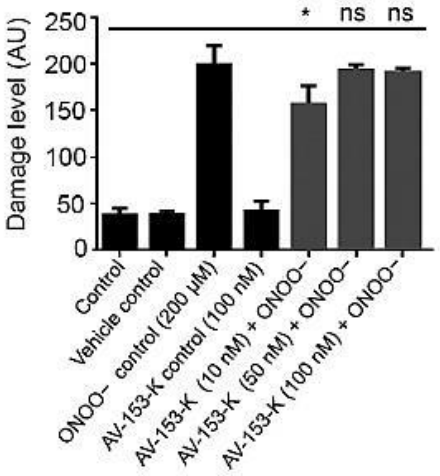
AV-153-Ca



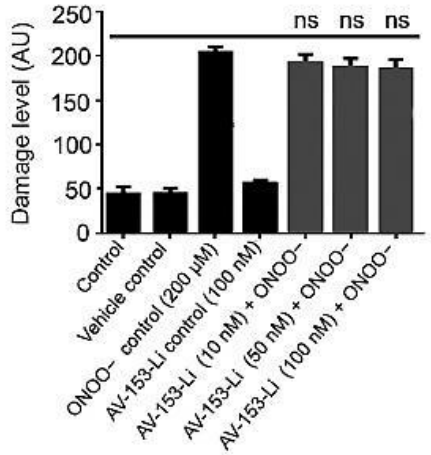
AV-153-Mg



AV-153-K



AV-153-Li



AV-153-Rb

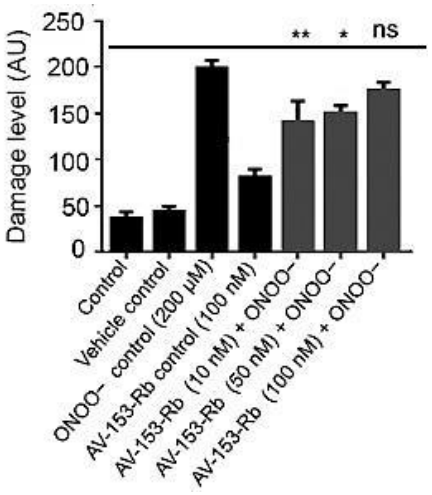


Figure 3.11 **Effects of AV-153 salts against peroxynitrite caused damage in Hela cell line tested by comet assay.** Cells were pre-incubated with different concentrations of AV-153-salts for 45 minutes, and then treated with peroxynitrite (bolus of 6 μL to a final concentration 200μM for 15 seconds). Cells were washed and treatment was repeated. Peroxynitrite was discarded, cells were washed and taken for comet assay. * - $p<0.05$ versus peroxynitrite group; **- $p < 0.01$ versus peroxynitrite group; *** - $p < 0.001$ versus peroxynitrite group; ****- $p < 0.0001$ versus peroxynitrite group; #-$p<0.05$ versus controlgroup; ns – not

significant. Experiments were repeated 3 times and data are presented as mean ± standart error of the mean (SEM).

3.7.2 Lipophilic derivatives of 1,4-DHPs

Some 1,4-DHPs with ability to interact with DNA and to scavenge the peroxynitrite were also tested for ability to protect cells againt peroxynitrite. Cerebrocrast could protect DNA of the cells against peroxynitrite. Hela cells treated with peroxynitrite alone and in the presence of cerebrocrast and etaftorone are presented in Figure 3.12 . Treatment with peroxynitrite drastically increase the level of DNA damage. Pre-incubation (Fig. 3.12) and simultaneous administration (Supplementary material Fig. 2A) with cerebrocrast both at 10, 50 and 100 nM produce significant and dose dependent effect. Pre- incubation with etaftorone was no efficient, however simultaneous incubation with etaftorone reduced DNA damage of the cell (Supplementary material Fig. 2B)

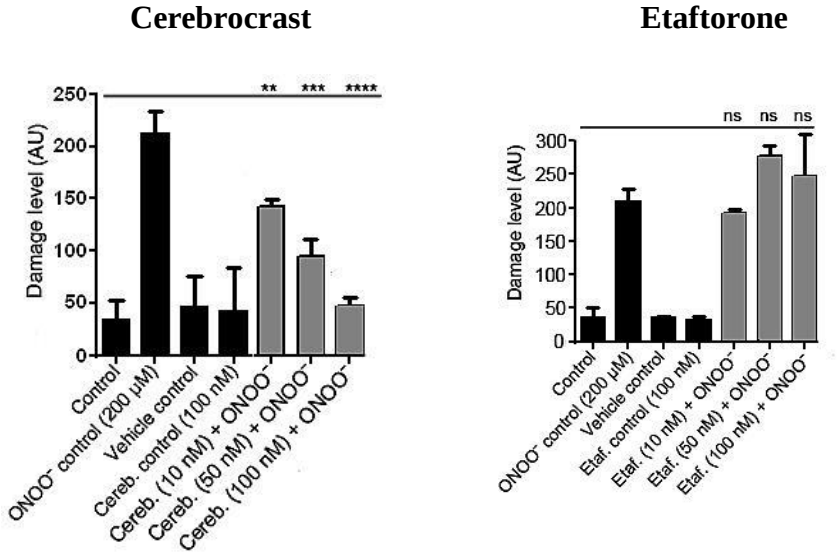


Figure 3.11 **Effects of cerebrocrast and etaftorone against peroxynitrite caused damage in HeLa cell line tested by comet assay.** Cells were pre-incubated with different concentrations of compounds for 45 minutes and then treated with peroxynitrite (bolus of 6 μL to a final concentration 200 μM for 15 seconds). Cells were washed and treatment was repeated. * - $p<0.05$ versus peroxynitrite group; **- $p < 0.01$ versus peroxynitrite group; *** - $p < 0.001$ versus peroxynitrite group; ****- $p <0.0001$ versus peroxynitrite group; ns – not significant. Experiments were repeated 3 times and data are presented as mean ± standart error of the mean (SEM).

3.8 DNA repair enhancement in the presence of AV-153-Na

The DNA repair in HeLa cells after peroxynitrite treatment was tested in DMEM alone and in the presence of AV-153-Na salt. Comet assay in alkaline conditions was used for these purposes. As presented in Figure 3.12, DNA repair rate significantly increased in the presence of AV-153-Na at the first 10 minutes in comparison with DMEM alone.

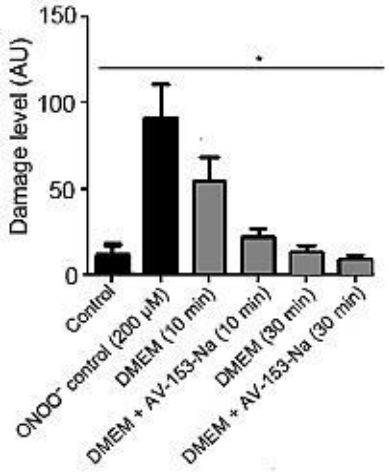


Figure 3.12 **The enhancement of DNA repair in the peresence of AV-153-Na.** After treatment with peroxynitrite, 50 nM of AV-153-Na was added to the medium and incubated for 10 and 30 minutes. * - $p<0.05$ versus peroxynitrite group; Experiments were repeated 3 times and data are presented as mean ± standart error of the mean (SEM).

3.9 DNA protecting effect of AV-153-Na against DUX4 pro-apoptotic protein

It was interesting to test impact of the AV-153-na on DNA damage triggered by other factors. DUX4 is a pro-apoptotic protein provoking oxidative stress in cells (Bosnakovski et al. 2008). Transfection of a pro-apoptotic protein DUX4 to HeLa cells significantly increased basic level of DNA damage. Comet assay in alkaline conditions was used for DNA damage detection. Incubation with AV-153-Na almost normalized the level of DNA breaks in transfected cells. Peroxynitrite-induced level of DNA breakage was also higher in transfected compared to non-transfected cells, incubation with AV-153 decreased it.

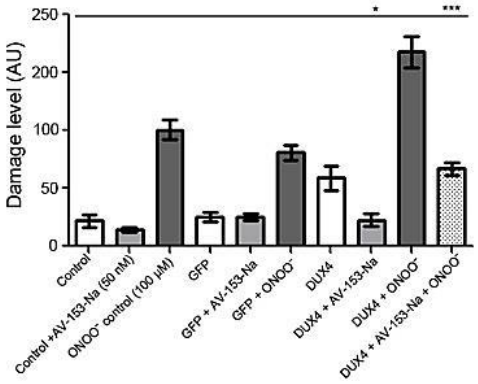


Figure 3.13 **DNA protecting effect of AV-153-Na in cell cultures against DUX4 protein.** GFP –transfection control. * - $p<0.05$ versus DUX4 group; *** -$p<0.001$ versus DUX4 + $ONOO^-$ group. Experiments were repeated 3 times and data are presented as mean ± standart error of the mean (SEM).

4. DISCUSSION

4.1 Dependence of affinity to DNA and binding mode on 1,4-DHP structure

In the current study we investigated DNA binding capacity and binding modes of water soluble and lipophilic derivatives of 1,4-DHPs which considered to be `unusual`. We have found that a number of representatives of 1,4-DHPs have and ability to bind DNA and it was revealed that the interaction of 1,4-DHPs with DNA molecule depends on their structural feature. Firstly we found out that monocyclic and water-soluble derivatives of 1,4-DHPs which contain ethoxycarbonyl groups in position 3 and 5 (AV-153 salts) have a high affinity to DNA. Previously it was shown that AV-153-Na interacts with DNA via intercalation in a place of single strand break (Buraka et al. 2014). However, testing the ability of different AV-153 salts to interact with DNA the unexpected differences in their binding constants were revealed. Affinity of the AV-153 salts to DNA was highly dependent on metal ion forming a salt in position 4. Binding constants of dimeric complexes of Ca and Mg salts were close to Na salt. Data of the binding site calculation pointed to intercalation of Na, Mg and Ca as a major binding mode, Li, K and Rb salts are sooner groove binders (Arshad et al. 2012, Shahabadi, Kashanian, and Darabi 2009). It should be taken into account that ions of metals in association with flexible 1,4-DHP ring existing in the “boat” conformation may cause conformational changes of molecule not favourable for penetration between DNA strands, but allowing external binding. 1,4-DHPs undergo transition between “boat” and planar conformation. Planar conformation is more rigid therefore favourable for intercalation. “Boat” conformation is more folded and typical for sticking to the groove (J Hofmann and Cimiraglia 1989). Ca, Na and Mg ions cause conformational changes of AV-153 and favour a more rigid planar conformation allowing penetrating between DNA strands, meantime other ions favour the “boat” conformation, favourable for groove binding. Moreover, results of EtBr displacement assay indicated the possibility for intercalation of the compounds between DNA strands, Ca and Mg extruded EtBr more intensively than others did. The ability of AV-153-Na to penetrate between DNA strands was confirmed by circular dichroism, the ICD signal of compound increased after every added DNA portion with a red shift pointing to intercalative binding mode (Garbett, Ragazzon, and Chaires 2007, Thimmaiah et al. 2015). FT-IR spectroscopy data determined the interaction of AV-153 salts with DNA-bases,

preferably with guanine and cytosine and DNA phosphate groups (Supplemetary material Fig. 3). However, the main changes were detected for absorption bands assigned to phosphate groups. UV/VIS data of AV-153-Na complexes with bases revealed a higher affinity to guanine.

Among another tested 1,4-DHPs we have also found out compounds with properties to interact with DNA. 1,4-DHP with propoxycarbonyl groups in position 3 and 5 (J-4-96) revealed a high affinity to DNA and binding constant was close to AV-153 group. J-4-96 has a low competitiveness with EtBr that is why it could points to groove binding as a major binding mode. The presence of acetyl groups in positions 3 and 5 (AV-154-Na) deprives the possibility of compound to interact with DNA. The addition of acetyl group to positions 2 and 6 (J-131-Na) also did not increase the affinity to DNA.binding site

PP-544-NH_4, the representative of tricyclic fused derivatives of 1,4-DHP-decahydroacridine-1,8-diones (PP-150-Na, B-5-Na, PP-544-Na and PP-544-NH_4) with ammonia group in position 4 proved itself as active DNA binder. Moreover it effectively extruded EtBr from intercalation sites, pointing to intercalation. Interestingly, the replacement of ammonia group in position 4 with sodium (PP-544-Na) took away the ability to bind DNA. Another modifications of tricyclic 1,4-DHPs molecule by the replacement of carboxylic group with aromatic ring in position 4 and addition the salt of caproic acid to nitrogen in position 1 (PP-150-Na) considerably decreased the effectiveness of DNA binding. However, after the addition of DNA to both PP-150-Na and B-5-Na solutions, emission maximum of compounds shifted to a red side, but without the fluorescence intensity changes, this effect could point to DNA-compound complex formation.

1,4-DHPs containing the amino acid moieties also have an ability to bind DNA, but seems not all of them. Addition of alanine in position 4 as amide of 2,6-dimethyl-3,5-dietoxycarbonyl-1,4-dihydroisonicotinic acid (alapyrone) abolished an ability to bind DNA, however taurine (tauropyrone) maintained the ability to interact with DNA probably via groove binding given the inability to compete with EtBr.

Styrylcarbatone (J-9-125) containing styryl group in position 4 manifested the ability to form a non-fluorescent complex (NFC) with DNA. Probably NFC could be formed through intercalation, because the enhanced fluorescence of the DNA-EtBr complex was remarkably quenched by this compound. Other carbatonides

(carbatone, metcarbatone, etcarbatone) lack the ability to bind DNA.

All lipophilic representatives of 1,4-DHPs (cerebrocrast, etaftorone and fenoftorone) have a difluoromethoxy group at position 2. Among them only cerebrocrast demonstrated the ability to interact with DNA molecule. Cerebrocrast form NFC with DNA. However, as opposed to PP-544-NH_4, it could be suggested, the NFC is formed through groove binding or semi-intercalation in connection with a weak impact on displacement of EtBr. Surprisingly, the structures of cerebrocrast and etaftorone are really similar, etaftorone has a shorter 2-propoxy-ethyl side chains, and however it negatively influenced the possibility to bind DNA. The addition of phenyl rings to the side chains of fenoftorone did not improve the DNA binding.

4.2 Interrelation of DNA binding and DNA protection

DNA binding usually considered being mutagenic and a high affinity to DNA might seem to be in contradiction with antimutagenic and DNA-protecting activities of the compound. However, there are literature data which prove the coherent effects of DNA-binding activity and antimutagenic effects. Prime example is a widely used antidiabetic drug metformin. It protects the type 2 *diabetes mellitus* patients from cancer development (Safe, Nair, and Karki 2018), it protects DNA against breakage (Algire et al. 2012), modify the processes of DNA repair (Lee et al. 2016) meantime it binds the DNA (Shahabadi and Heidari 2012).

We have tested AV-153 salts and others 1,4-DHPs for the ability to protect DNA against peroxynitrite induced damage. Before that it was detected Mg, Ca and Li salts have an ability to react with peroxinitrite. It seems the presence of divalent ions in solution could accelerate the decomposition rate of peroxynitrite. However, this direct interaction with peroxynitrite is not the main DNA-protecting mechanism of these compounds. According to the results of comet assay Mg salt was the worst DNA protector against oxidative stress compared with Ca and Na salts. Moreover, AV-153-Mg *per se* increased damage level of DNA. Pre-incubation with several AV-153 salts significantly decreased DNA damage, simultaneous incubation was not effective; this again indicates the importance of intracellular processes, but not direct but chemical reaction, for DNA protection. These results illustrate the duality of action of some these salts; they both induce DNA damage and decrease these effects of other DNA-damaging agents at the same time. The higher efficiency of low concentrations of

AV-153 salts observed in some cases could reflect a shift of the equilibrium between DNA damage and DNA protection towards DNA damage. The dimeric Mg salt, possessing the highest affinity to DNA may produce excessive DNA damage; this is observed as DNA breakage produced by the compound itself and low DNA protection efficiency. A necessity for pre-incubation and lower efficiency of simultaneous administration with peroxynitrite indicates that AV-153 salts induce some changes in the cells favouring protection of DNA or DNA-repair, as these compounds bind peroxynitrite with low affinity. Moreover, the good DNA binder, AV-153-Ca was the most effective DNA protecting agent, AV-153 salts with lower DNA binding constants (AV-153-Li, AV-153-K) were worse protectors. The similar pattern emerges in case with lipophilic derivatives of 1,4-DHP where the cerebrocrast, which is an active DNA binder, after pre-incubation significantly and dose-dependent protected DNA in living cells against peroxynitrite induced damage. Etaftorone which has no affinity to DNA was not effective. However, cerebrocrast and etaftorone are perfect peroxynitrite scavengers and both was effective reducing DNA damage after simultaneous incubation. It could be suggested that cerebrocrast has a dual mechanism of action, namely it protects DNA by scavenging of oxidative molecules and binding directly to DNA. DNA binding studies study rased a question about the possibility of the compounds to reach DNA. Using laser confocal microscopy we have found that in the cell the good DNA binder AV-153-Na mostly is accumulated in cytoplasm; however, it has an ability to reach nucleus (Supplementary material Fig. 4). Stained cytoplasmic structures raised the question about protein binding that was proved later using fluorescence assay and circular dichroism. It was suggested that cytoplasmic proteins retain the main part of compound after exposure of the cell and only a small part reaches DNA, where it activates DNA repair systems but does not produce harmful effects due to very low concentration of the compound in the nucleus. Also we have found that AV-153-Na enhanced DNA repair after peroxynitrite treatment. The data on the impact of AV-153-Na on the activity of the excision repair enzymes makes understanding of the mechanism of action of the compound possible. It was found that AV-153-Na triggers an increase in activity of DNA repair enzymes, mostly performing excision/synthesis repair of 8-oxoguanine (8-oxoG), abasic sites (AP sites) and alkylated bases (Supplementary material Fig. 5). Thus binding of AV-153-Na to DNA could increase the activity of DNA repair

enzymes, in low doses, possible lesions produced by the compound itself are steadily repaired, but mobilization of the repair systems favours a higher resistance to other genotoxic agents.

5. CONCLUSIONS

1. Affinity of the 1,4-DHP derivatives to DNA depends on groups in positions 3, 4 and 5. Compouds with carboxyluic group in position 4 and ethoxycarbonyl groups in positions 3 and 5 (AV-153) manifest the highest affinity. Salts of AV-153 with different metal ions in position 4 interact with DNA with different affinity decreasing in following order Mg>Na>Ca>Li>Rb>K. Tricyclic 1,4-DHP derivatives are also good DNA binders. Among investigated lipophilic compounds, only the cerebrocrast effectively interacts with DNA.

2. Both intercalation and groove binding are possible modes of 1,4-DHP and DNA interactions. Among AV-153 salts Na, Mg and Ca are sooner intercalators, but Li, K and Rb salts are groove binders. Styrylcarbatone (J-9-125) and PP-544-NH_4 are intercalators, but J-4-96 and cerebrocrast are groove binders.

3. AV-153-Na can interact with the four types of bases, with somewhat different affinities. Affinity to G, C and T is greater than that to A.

4. Fluorescence titration and circular dichroism showed that AV-153-Na have a high affinity to human serum albumin. AV-153-Na has one independent binding site per one HSA molecule. Other salts of AV-153 also interact with HSA.

5. Mg, Li and Ca salts of AV-153 cause peroxynitrite decomposition, others do not. Other water soluble 1,4-DHPs also do not react with peroxynitrite. Hovewer, all lipophilic derivatives of 1,4-DHP cerebrocrast, etaftorone, fenoftorone enhance the breakdown of peroxynitrite. Probably PP-544-NH_4 forms a complex with peroxynitrite.

6. Pre-incubation with several AV-153 salts significantly decreased DNA damage, simultaneous incubation was not effective. AV-153-Mg *per se* increased damage level of DNA. The good DNA binder, AV-153-Ca and AV-153-Na are the most effective DNA protecting agents, AV-153 salts with lower DNA binding constants (AV-153-Li, AV-153-K) are not so effective.

6. APROBATION OF RESEARCH
LIST OF PUBLICATIONS AND PRESENTATIONS

I. **Buraka,** E., C. Y. Chen, M. Gavare, M. Grube, G. Makarenkova, V. Nikolajeva, I. Bisenieks, I. Bruvere, E. Bisenieks, G. Duburs, and N. Sjakste. (2014). "DNA-binding studies of AV-153, an antimutagenic and DNA repair-stimulating derivative of 1,4-dihydropiridine." Chem Biol Interact 220:200-7. doi: 10.1016/j.cbi.2014.06.027.

II. **Leonova,** E., J. Sokolovska, J. L. Boucher, S. Isajevs, E. Rostoka, L. Baumane, T. Sjakste, and N. Sjakste. (2016). "New 1,4-Dihydropyridines Down-regulate Nitric Oxide in Animals with Streptozotocin-induced Diabetes Mellitus and Protect Deoxyribonucleic Acid against Peroxynitrite Action." Basic Clin Pharmacol Toxicol 119 (1):19-31. doi: 10.1111/bcpt.12542.

III. **Leonova E**, Rostoka E, Sauvaigo S, Baumane L, Selga T, Sjakste N. Study of interaction of antimutagenic 1,4-dihydropyridine AV-153-Na with DNA-damaging molecules and its impact on DNA repair activity. PeerJ. 2018 Apr 25;6:e4609. doi: 10.7717/peerj.4609. eCollection 2018.

IV. **E. Leonova**, E. Rostoka, L. Baumane, V. Borisovs, E. Smelovs, I. Bisenieks, I. Brūvere, E. Bisenieks, G. Duburs and N. Sjakste DNA-binding studies of a series of novel water-soluble derivatives of 1,4-dihydropyridine Biopolymers and Cell. 2018. Vol. 34. N 2. P 129–142

The data are also presented in the congresses and conferences and published is abstracts

1) Buraka, E. Yu-Chian Chen, C, Nikolajeva, V., Grube, M., Gavare, M, Duburs, G. and Nikolajs Sjakste. *Antimutagenic and repair – stimulating derivative of 1,4 – dihydropiridine AV -153 intercalates in DNA in a single strand break site between two pyrymidines.* Debrecen University Symposium 2013, 23rd Wilhelm Bernhard workshop on the cell nucleus. 19-24 August, Hungary. In book of abstract (77.p)

2) E.Rostoka, S.Isajevs, **E. Buraka,** L. Baumane, N. Sjakste. *Search for molecules with combined antioxidant and DNA-binding activities.* Ecole Nationale Supérieure de Chimie de Paris. From Molecular to Cellular events in Human Pathologies. 17-18 October, Paris, France

3) Jelizaveta Sokolovska, Evita Rostoka, **Elīna Buraka**, Olga Sudoka, Sergejs Isajevs, Larisa Baumane, Jelena Sharipova, Ivars Kalvins, Nikolajs Sjakste *Nitric oxide Hyperproduction and markers of DNA damage in the early phase of diabetic nephropathy in rat streptozotocin diabētus mellitus model.* 27th European diabetic nephropathy Study Group Meeting, 2014, 16-17 May, London

4)Evita Rostoka, **Elina Buraka,** Larisa Baumane, Jean-Luc Boucher, Vitalijs Borisovs, Nikolajs Sjakste *In vitro studies of 1,4-dihydropyridine peroxynitrite scavenging and DNA protective activities*. The 2nd Genetics and Genomics Conference, June 13-15, 2014, Beijing

5)**Elīna Buraka**, Evita Rostoka, Vitālijs Borisovs, Gunārs Duburs, Nikolajs Sjakste *1,4-DHP Atvasinājumu Antioksidatīvo Īpašību Un To Mijiedarbības Ar DNS Analīze*. The 56th

International Scientific Conference Of Daugavpils University. April 9-14, 2014, Daugavpils, Riga Oral Presentation

6) **Elina Buraka,** Evita Rostoka, Gunars Duburs, Nikolajs Sjakste. *Evaluation of peroxynitrite-scavenging and DNA binding capasiyu of several 1,4-dihydropiridine derivatives using spectroscopy and single-cell gel electrophoresis.* 14th ISANH Congress on Oxidative Stress Reduction, Redox Homeostasis and Antioxidants, June 12-13, 2014, Paris Poster. In book of abstract (88.p)

7)Elina Buraka, Rostoka Evita, Duburs Gunars, Sjakste Nikolajs. *Acceleration of the peroxynitrite decomposition rate, DNA binding and oxidative stress reduction by 1,4-dihidropiridine derivatives.* VII International Meeting From Molecular to Cellular Events in Human Pathologies. 17-20 October 2014, Riga, Latvia. Oral presentation. In book of abstract (7.p)

8) Elina Leonova, Evita Rostoka, Yara Bou Saada, Yegor Vassetzky ,Gunars Duburs, Nikolajs Sjakste. Affinity to DNA and other activities of a series of 1,4-DHP. Drug Discovery Conference. 27-29 August, 2015, Riga, Latvia. Oral presentation. In book of abstract (78 p.)

9) **Elina Leonova,** Evita Rostoka, Sylvie Sauvaigo ,Larisa Baumane , Vitalijs Borisovs, Yara Bou Saada, Egils Bisenieks, Yegor Vassetzky, Gunars Duburs and Nikolajs Sjakste. Oxidative stress reduction and DNA repair enhancement by 1,4-dihydropiridine AV-153. The 58th International Scientific Conference of Daugavpila University. 14-15 April, 2016. Daugavpils, Latvia. Oral Presentation. In book of abstract (99 p.)

Local conferences and congresses:

1) E. Buraka, E. Rostoka, G. Duburs, N. Sjakste. 1,4 - DHP effect on the rate of peroxynitrite decay process and their ability to protect cells from DNA damage caused by peroxynitrite. 72. Scientific conference of University of Latvia 2014. Oral presentation. In book of abstract (24-25 pp.)

2) E.Leonova, S. Sauvaigo, Y.Bou Saada, V. Borisovs, E. Rostoka, E. Bisenieks, Y. Vassetzky, G. Duburs, N. Sjakste. The impact of antimutagenic 1,4-dihydropiridine AV-153-Na on DNA repair activity. 74.Scientific conference of University of Latvia 2016. Oral presentation.

3) **Elina Ļeonova,** Evita Rostoka, Sylvie Sauvaigo, Vitalijs Borisovs, Egils Bisenieks, Yegor Vassetzky, Anna Tolstova, Nikolajs Sjakste. 1,4-dihydropyridines: mechanism of action. 75. Scientific conference of University of Latvia, January 2017, Riga, Latvia. Oral presentation

4) Edgars, Smelovs, **Elīna Ļeonova,** Nikolajs Sjakste. Study of antioxidant properties and interactions with dna of 1,4-dihydropyridine derivatives using spectroscopic methods. 75. Scientific conference of University of Latvia, 24 February, 2017, Riga, Latvia. (In book of abstract)

5) **Elīna Ļeonova**, Nikolajs Sjakste Dna Lesions In Healthy Subjects: A Literature Review. 75. Scientific conference of University of Latvia, 24 February, 2017, Riga, Latvia. Oral Presentation (In book of abstract)

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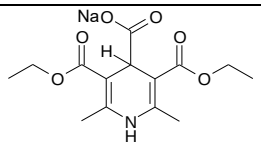
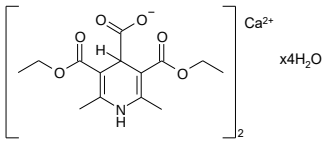
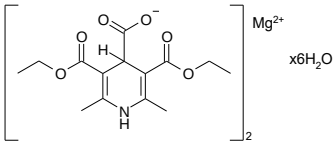
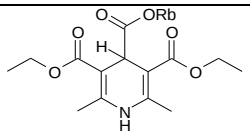
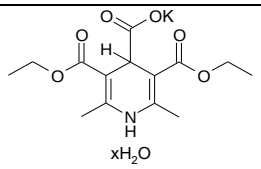
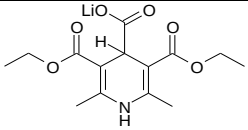
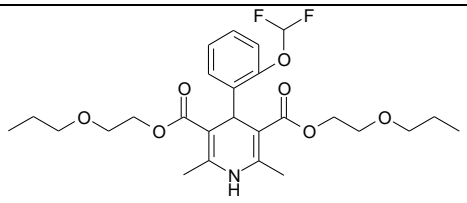
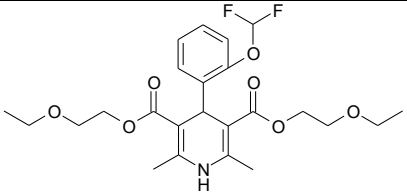
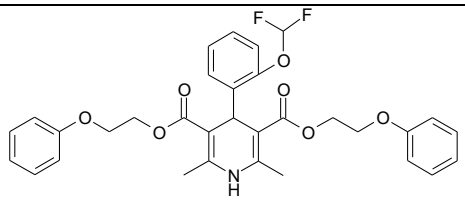
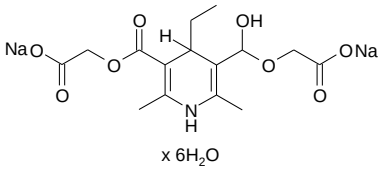
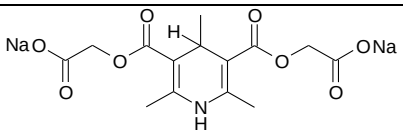
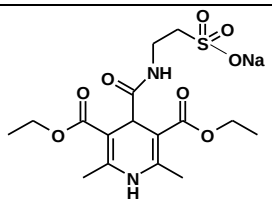
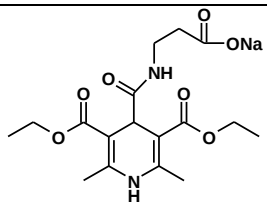
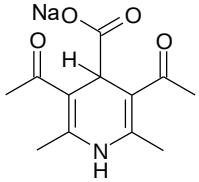
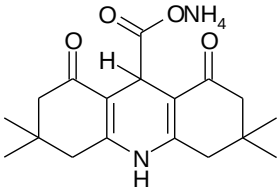
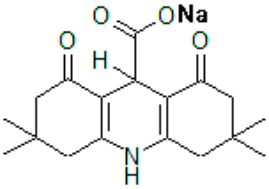
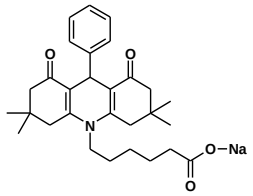
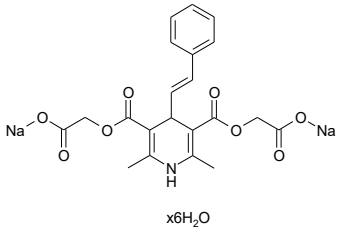
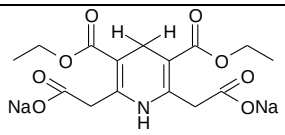
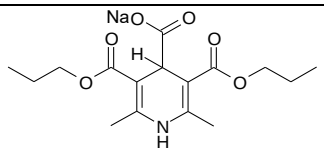
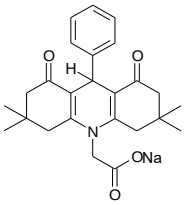
9. SUPPLEMENTARY MATERIAL

Table 1

Chemical structures of tested compounds

Compound	Chemical structure	MW [g/mol]
AV-153-Na		319.3
AV-153-Ca		704.77
AV-153-Mg		724.99
AV-153-Rb		381.77
AV-153-K		353.4
AV-153-Li		303.2
Cerebrocrast		511.56
Etaftorone		483.5

Fenoftorone		579.60
Etcarbatone	 x $6H_2O$	493.37
Metcarbatone	 x $6H_2O$	479.34
Tauropyrone		426.42
Alapyrone		390.36
AV-154-Na		259.2
PP-544-NH_4		334.4
PP-544-Na		403.4

PP-150-Na		485.59
J-9-125 (Styrylcarbatone)		567.50
J-131-Na		421.3
J-4-96		356.3
B-5-Na		429.5

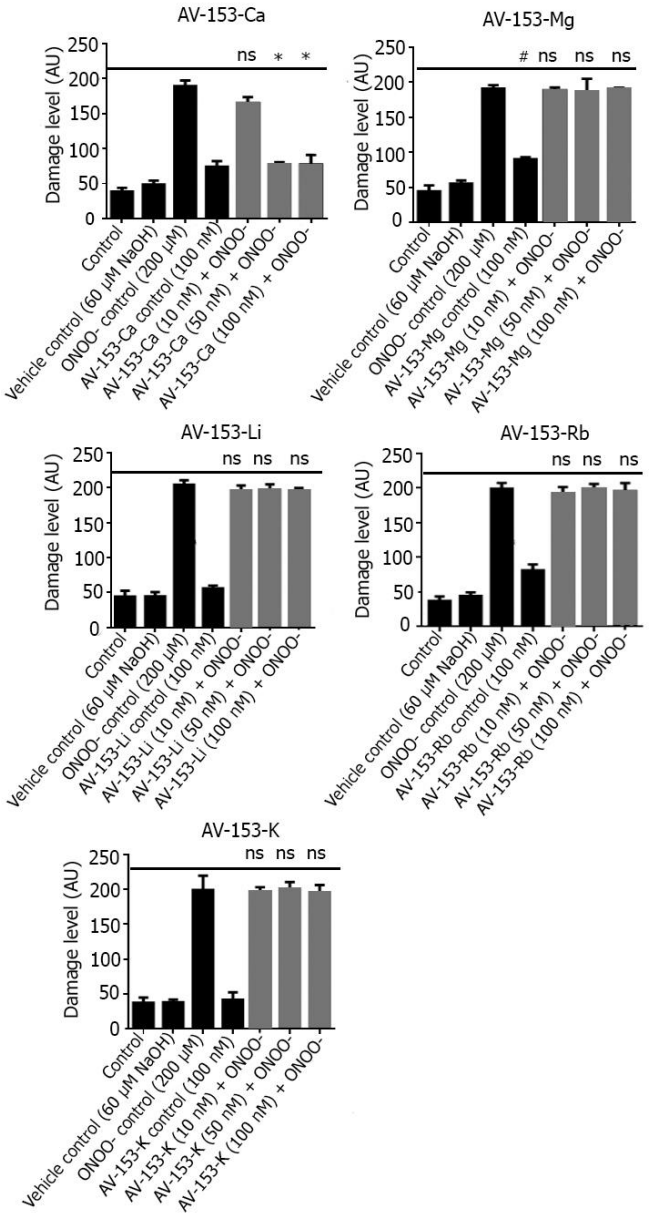


Figure.1 **Simultaneous incubation effects of AV-153 salts against peroxynitrite caused damage in Hela cell line tested by comet assay.** Cells were simultaneously treated with different concentrations of AV-153 salts and peroxynitrite (bolus of 6 μL to a final concentration 200μM for 15 seconds). Cells were washed and treatment was repeated. Peroxynitrite was discarded, cells were washed and taken for comet assay. * - $p<0.05$ versus peroxynitrite group; **- $p < 0.01$ versus peroxynitrite group; *** - $p < 0.001$ versus peroxynitrite group; ****- $p <0.0001$ versus peroxynitrite group; #-$p<0.05$ versus controlgroup; ns – not significant. Experiments were repeated 3 time and data are presented as mean ± standart error of the mean (SEM).

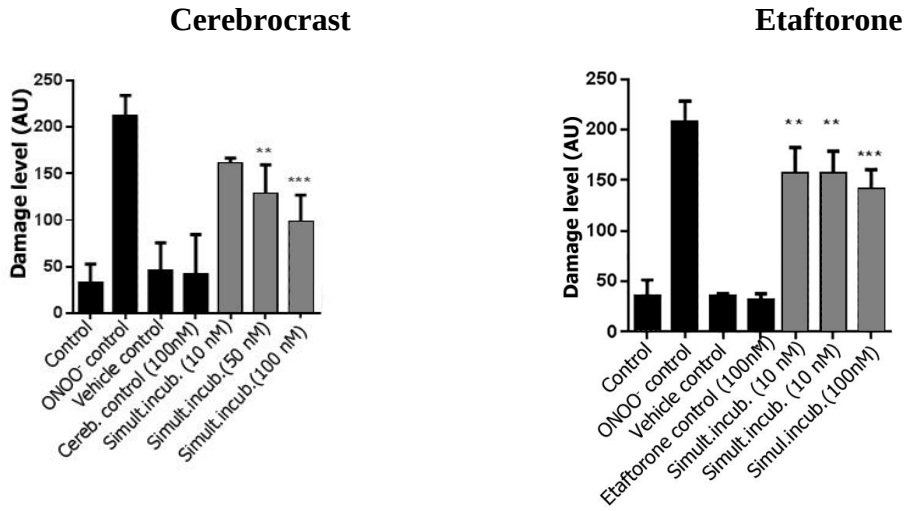


Figure 2. **Simultaneous incubation effects of cerebrocrast and etaftorone against peroxynitrite caused damage in Hela cell line tested by comet assay.** Cells were simultaneously treated with different concentrations of compounds and peroxynitrite (bolus of 6 μL to a final concentration 200μM for 15 seconds).. Cells were washed and treatment was repeated. Peroxynitrite was discarded, cells were washed and taken for comet assay. * - $p<0.05$ versus peroxynitrite group; **- $p < 0.01$ versus peroxynitrite group; *** - $p < 0.001$ versus peroxynitrite group; ****- $p <0.0001$ versus peroxynitrite group; #-$p<0.05$ versus controlgroup; ns – not significant. Experiments were repeated 3 time and data are presented as mean ± standart error of the mean (SEM).

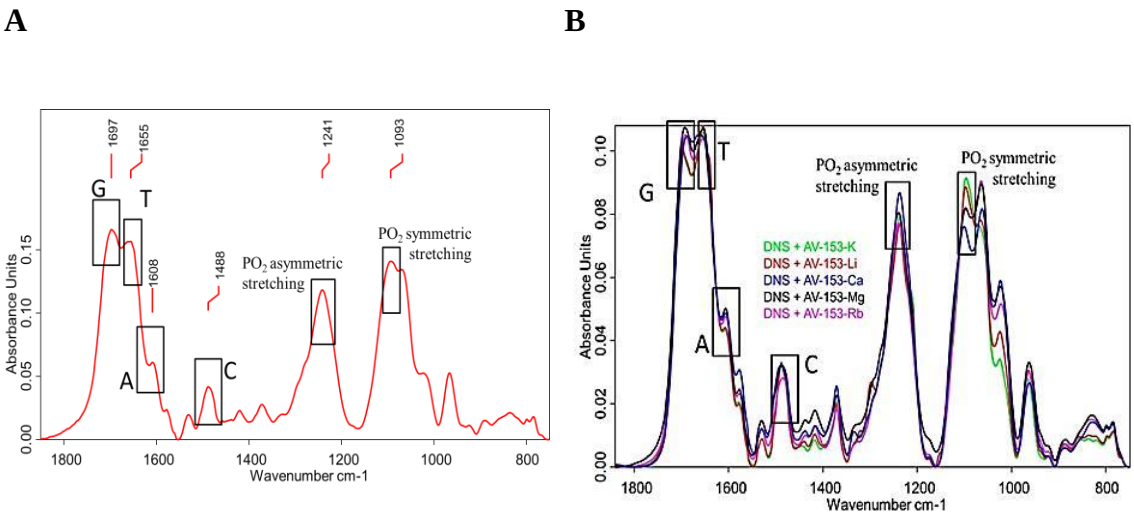


Figure 3. **FTIR spectra ct-DNA with marked absorptions of DNA bases and PO_2 groups (A); FT-IR spectra of DNA with AV-153-salts (5:1) (B).**

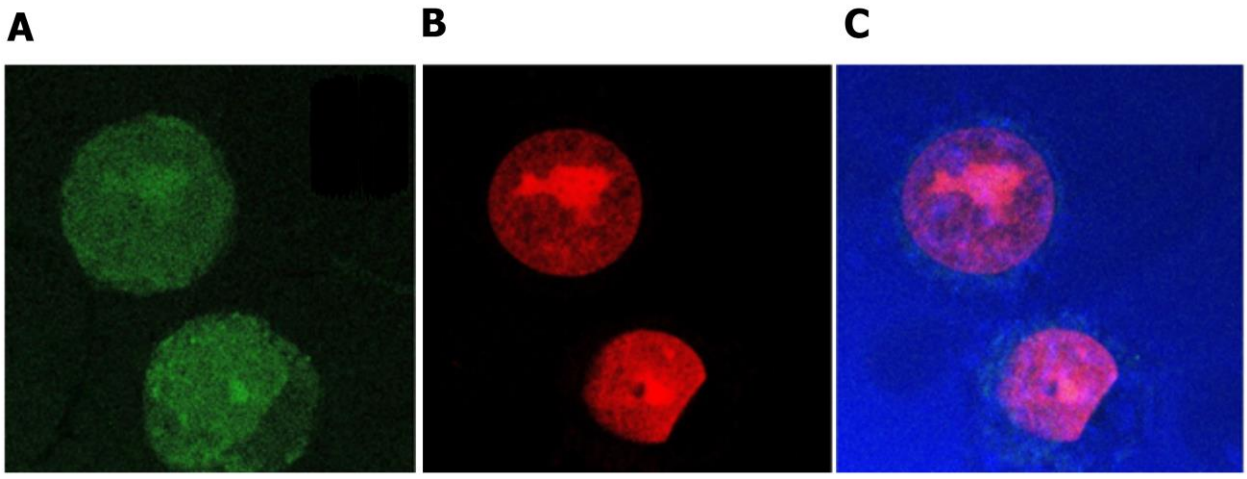


Figure 4. **AV-153-Na localization in the cell.** (A-C) images of Hela cells which were treated with AV-153-Na, DNA was stained with propidium iodide (PI). Blue light – reflected light; (A) green-distribution of AV-153-Na (B) red- propidium iodide; (C) the overlay image of all channels.

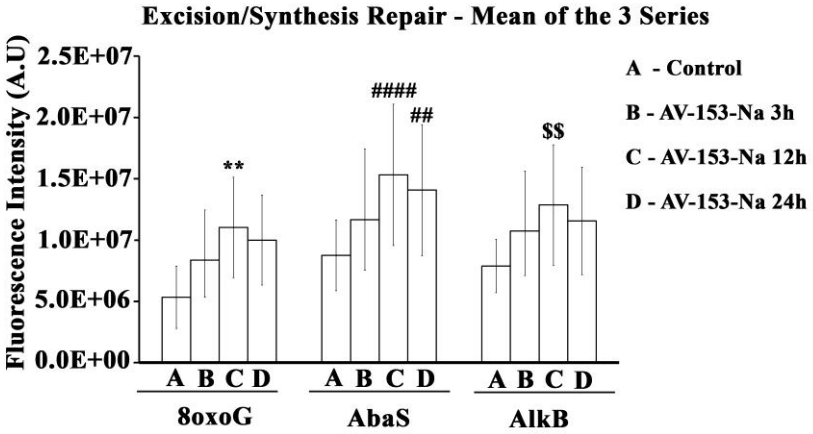


Figure 5. **Effect of AV-153-Na on cellular Excision/Synthesis Repair (ExSy-SPOT assay) of major base lesions.** The repair reaction was conducted with nuclear extracts prepared from non-treated cells and cells treated for 3h, 12h and 24h with AV-153-Na. For each lesion, we calculated the ratio of the fluorescence intensity obtained with the treated cells over the fluorescence intensity obtained with the control cells. **p<0.01 versus 8oxoG control; ####p<0.0001 versus AbaS control; $$p <0.01 versus AlkB control